

Avoiding academic injustice in Saxony

Post-war Germany's enviable reputation as a law-abiding state is threatened by the harsh treatment of a handful of academics in the eastern *Länder* who once belonged to the Communist Party of the German Democratic Republic.

DR Armin Ermisch is still excluded from his post as head of the Department of Cell Biology at the University of Leipzig more than a year after he was first dismissed from it and in spite of his successful appeal to a German court. He was dismissed a second time in June this year. His appeal against that administrative decision will be held late in November. His post is no longer vacant, but occupied by a successor appointed before the first appeal had been adjudicated. It is a distressing tale and a haunting one, with all the makings of a serious injustice. It is also a cruel reminder of the damage that can follow from even beneficent events, in this case the reunification of Germany at the end of 1991.

Ermisch is a distinguished scientist, both in the old German Democratic Republic (GDR) and internationally. Like all academics at the University of Leipzig, his terms of employment are determined by the new *Land* of Saxony. As part of the reunification contract, Saxony and the other eastern *Länder* set up commissions to rule on the "personal integrity" of public employees, academics included (see *Nature* 359, 762; 1992). The first time round, Ermisch was one of many academics who were sent dismissal notices. His listed various discreditable behaviours, included complicity with the notorious secret police known as the *Stasi*, in particular informing on his academic colleagues, and the abuse of his position as head of a university department. Ermisch's appeal court exonerated him on all these counts and recommended reinstatement.

The second dismissal notice, delivered in June, cites two other grounds: membership of the Communist Party and (in that role) the provision of advice to students (at the end of 1990) that they should not take part in the street demonstrations that first suggested that even the apparently implacable GDR was not immune to change. Ermisch's fresh appeal consists of two parts: the argument that the advice he gave his students is a measure of his concern for their safety, and that his membership of the Party was an indispensable, but otherwise meaningless, qualification for the head of a university department.

The Ermisch case is not, of course, the only one to have arisen since reunification. A year ago, hundreds of appeals by academics against dismissal were in the eastern *Länder* courts. Not all of them were resolved in favour of the appellants, many of whom (and others, who had not appealed) found it easier (and possible) to find jobs elsewhere and move. Ermisch, well-known as he is, could probably do the same, although (in his fifties) probably only with some

difficulty and disadvantage. But he appears to want to stay where he is, where over the years he has trained many able graduate students.

If the Ermisch case was an isolated illustration of how, during great social upheavals such as that of reunification, some degree of rough justice is unavoidable, it could be overlooked. But it is not. And the underlying issues of principle have not been faced as squarely as Germany's reputation would lead outsiders to expect. At this stage, of course, nobody pretends that the GDR was a democratic state or that the Communist Party was not both its chief means of coercion and the chief beneficiary of that state of affairs. But it is an assault on reality to suppose that all those who belonged to the old Party were equally responsible for its malign influence. That would be to suppose that the Party was not also coercive of its own members and to deny the simple truth of experience that membership became a duty for those holding certain public offices just as it was originally a privilege for others. Saxony seems to have put aside both these arguments.

Several questions then arise. It is (or it should be) readily appreciated that people whose lives have been perverted for a generation should be bitterly resentful of the instruments of their past torment, but will not insensitive retribution engender further bitterness? It is especially dangerous when academic institutions witness acts of injustice such as that at Leipzig, for universities have a special duty of tolerance. And, given the likelihood that an appeal by Ermisch to the federal Constitutional Court at Karlsruhe would almost certainly succeed, should not Saxony give in now, before causing further embarrassment for itself? □

Travel loosens tongues

Britain's prime minister has acknowledged in Japan what he would not in Britain — that British science has been mishandled.

TRAVEL does not always broaden the mind (it can also be a reinforcement of prejudice), but the visit of the British prime minister, Mr John Major, to Japan last week appears to have had one beneficial effect. Major is the inheritor of a government which, over nearly 15 years, has dealt insensitively, even foolishly, with British science, substituting for what may previously have been unfounded optimism a general demoralization. The British research community now waits



to see whether the new arrangements outlined in the early summer will restore its spirits or instead, by linking most public research support to the goal of "wealth-creation", will drive the iron deeper. So it is some comfort that Major, when in Japan, accepted that British policies on research have not always been wise. "Perhaps", he said at an exhibition of the technical wonders of modern Japan, "we have undervalued science and the application of science over the last 20 or 30 years."

Major's timing was immaculate. He was speaking on the eve of this year's conference of the British Labour Party, which began on Monday. On that occasion 30 years ago, the then Mr Harold Wilson (he is now a Lord) offered to transform the British economy by "white-hot technology" and went on to win a general election the following year. Unfortunately, that promise came to nothing. After the Wilson government had arranged for the building of a few aluminium smelters and the amalgamation of several small companies into larger groupings, the forced devaluation of sterling stopped it in its modernizing tracks. No succeeding politician has dared echo Wilson's recipe for prosperity.

Does Major's mild remark signal that the tide is turning? It is something that he should have acknowledged the possibility of error. Even if this was no more than the familiar loosening of tongues occasioned by foreign travel, it is more like a plus than a minus. And although the statement directs attention to the more distant past, there is little doubt that it will be read in Britain as an acknowledgement of recent error also. If the administration in the past decade had been that admirable, would this summer's new policy have been necessary? Indeed, the new doctrine is in some respects Wilsonian: research harnessed to "wealth-creation". We shall soon learn what that means.

Wisely, Major did not confuse his Japanese audience with too much detail, but in one respect he seemed to have it thoroughly flummoxed. Noting that the new British policy for research has as its centrepiece a scheme for deciding what kinds of projects to back by means of a "technological foresight initiative", he praised Japanese efforts of that kind, saying that Britain would follow suit. People in Japan are still wondering what Major meant. It is true that the Science and Technology Agency in Tokyo publishes an annual list of guesses about future discoveries (cancer cured by 2003, thermonuclear fusion economic by 2020, that sort of thing), but that is not a recipe for research policy.

The best guess is that Major may have found among his briefing papers the legend that the Ministry of International Trade and Industry guides Japanese industry in directions determined by divination akin to the foresight technique that suddenly became fashionable in Whitehall a few months ago. The minister in charge of science, Mr William Waldegrave, is this week following Major to Japan in the hope of learning what foresight is before the time comes to practise it. The difficulty is that there is no such thing, but only what Adam Smith called the invisible hand, the often cruel censorship of manufacturers' ambitions by the forces engendered by free-spending and prosperous consumers. Major should not be too disappointed when Waldegrave returns empty-handed. □

What's in a name?

This week sees the appearance of yet another new section of *Nature*, under the somewhat old-fashioned title "Progress".

WHAT follows is an explanation of why this journal, which already has more named sections than can appear every week, should invent another. It is to be hoped that readers will not take this to be an attempt further to confuse them, but rather as a serious attempt to provide an account of progress (whence the name) in newly emergent fields of research. The purpose of articles appearing under this rubric is to draw attention to research topics only recently recognized as having an identity of their own and as potentially fruitful. They will ordinarily be written by practitioners in the field concerned, and will often (of necessity) describe research in which the authors have been personally concerned. For the convenience of readers, they will be squeezed within a maximum of four pages. They will supplement in an interesting way the cumulative record of discovery now provided by the standard weekly diet of original research and the commentary on it to be found under the heading "News and Views". They are not substitutes for formal reviews, but an alternative to them.

Every serious science journal, of course, takes it as given that one of the most valuable functions of the literature is to review emergent fields of research. By that means, the argument goes, students and others entering a field unfamiliar to them are provided with an account of what has gone before. By the same means, the research community is provided with a fair account of who did what, when and even why. And posterity (at least in the narrow sense of "people a few years from now") is provided with a means of understanding the origins of important avenues in research. All these are laudable objectives. Every active researcher applauds them. But most researchers are too busy to divert their energy and time to the compilation of a review of their own field. The need judiciously to apportion credit for intellectual innovation appears to be another impediment.

Those circumstances no doubt explain why the database of "reviews in preparation" patiently maintained by *Nature's* Reviews Coordinator is, on the face of things, enough to keep *Nature* amply supplied with reviews for several years ahead. The most common annotation of the database is "WRITING"; many authors, it emerges, are still "WRITING" several years after agreeing to do so. Some forgiving hearts bleed for the pain thus caused. Others, harder and perhaps more cynical, take this phenomenon to be yet another sign of the gulf between hope and reality in most human affairs. When President Boris Yeltsin's hope of containing Russia's money supply is so regularly defeated by his and his people's wish that there should be plenty of money to spend, why should *Nature* (like all other journals) be dismayed that so great a proportion of promised reviews fails to materialize? It will be excellent if the rubric "Progress" seems to authors so much less forbidding that they are able to turn "WRITING" into something else more quickly. □

Europe studies contingency plans for withdrawal from space station

Paris. Europe's plans to participate in the international space station being planned by the US National Aeronautics and Space Administration (NASA) were placed in doubt this week when officials from the European Space Agency (ESA) announced that the agency is thinking of redesigning its planned hardware contributions so that they could be used independently of the station.

If the proposal is accepted by the ESA council, the development of the agency's main contribution to the space station, an attached pressurized module (APM), would be virtually frozen until ESA ministers decide on its fate at their next meeting, due to take place in February 1995.

The need for cutbacks has been precipitated by the difficulties faced by several ESA member states in meeting their contributions to the agency's programmes. Speaking in Paris this week, Jean-Marie Luton, the agency's director-general, explained that

something has to give, and that the pressurized module is the "only candidate".

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Jean-Marie Luton
"something must give"

the move is growing uncertainty over Europe's role in the international space station Alpha — the name of final redesign of space station Freedom — and the consequent need for the European agency to make

By freezing further development work on the module until 1995, and by making design changes, ESA would be able to cut its cost over the period 1994–2000 from Ecu 1.82 billion (US\$ 2.2 billion) to Ecu 1.32 billion.

But the main reason for propos-

ing the move is growing uncertainty over Europe's role in the international space station Alpha — the name of final redesign of space station Freedom — and the consequent need for the European agency to make

contingency plans. Following an agreement announced earlier this month, the United States now seems likely to drop its plans to build Alpha, and instead combine it with Russia's planned MIR2 station to create a single "Global Space Station (GSS)". The GSS is likely to have an orbit at an inclination of 51.6 degrees, rather than the 28.2 degrees originally planned for Freedom.

Europe's participation in Alpha was assured to some degree under an intergovernmental agreement. Romano Barbero, head of the APM programme at ESA, says the United States acknowledges that it must consider the large Russian participation as an extension of its original agreement with its international partners in Alpha. He says that ESA will be negotiating to "safeguard our rights and costs as they are at the moment".

At the same time, Luton warns that Europe would be forced to withdraw from the programme unless the United States agrees to renegotiate ESA's contribution to the running costs of a station. Reinforcing this message, ESA's new plans for its pressurized module, as well as for spacecraft being developed for use with the station, envisage redesigning them so that they can be used in "a scenario without any significant participation in an international space station".

The mechanical and electronic aspects of the pressurized module would be redesigned so that it could be operated from Europe, says Barbero. It would also be reduced in size so that it could be put into orbit using the planned European Ariane V rocket, instead of NASA's space shuttle.

ESA's ministers will decide whether to proceed with the redesigned APM at their next meeting in February 1995. If the redesign does go ahead, the module would be launched in 2002, three years later than planned. The ministers will decide at the same meeting on the future of two non-reusable crew and cargo vehicles from the Hermes programme.

European hesitancy over the shape of the new, global space station will add to renewed concerns in Washington about the lack of detail which has emerged on it so far.

US funding for the space station is secure for another year after the Senate last week followed the House and voted down an attempt by Dale Bumpers (Democrat, Arkansas) to kill the programme. But four influential station supporters in Congress have written to vice-president Al Gore expressing their concerns about its impact on US jobs and on existing agreements with Canada, Europe and Japan. **Declan Butler**

US keeps out of THORP debate

Washington. The Clinton administration has decided to steer clear of the debate over whether Britain's Thermal Oxide Reprocessing Plant (THORP) at Sellafield in Cumbria should be allowed to start operation, thus defying pressure from environmentalists and some Congressmen.

In revealing his policy on nuclear non-proliferation to the United Nations in New York on Monday, President Bill Clinton concentrated on the need to control military plutonium and missile delivery technology. He ignored calls for the United States to come out against the opening of civil reprocessing facilities — such as THORP — which produce plutonium.

The British government's consultation period on the future of THORP ends on October 4, and opponents of the plant have been pushing for input from the US to that process to reinforce their opposition.

Earlier this month, for example, the House of Representatives passed a "sense of Congress" motion put forward by Joe Kennedy (Democrat, Massachusetts) stating that "the start-up or continued operation of any plutonium separation plant ... should be suspended until related environmental and proliferation concerns have been resolved."

Kennedy has also given his support to a bill introduced by Pete Stark (Democrat, California) which urges the President to take the issue to the UK government "in high-level bilateral discussions" before its

review of THORP is completed. The bill points out that THORP will produce 59 tonnes of plutonium in ten years, to add to the world's existing stockpile of 910 tonnes. But it stands little chance of becoming law.

Recognizing that time is short, 31 members of Congress signed a letter to Clinton late last week urging him to enter "immediate consultations with Britain, urging that Thorp not begin operations". But White House officials say that their position reflects the State Department's view that the THORP decision is a matter for the British government and its main customers in Germany and Japan.

Critics of that position argue that under the US-Japan nuclear cooperation agreement of 1988, the United States is ultimately responsible for the large, Japanese portion of THORP's throughput, and is therefore entitled to a say in the THORP decision.

They claim backing from the non-proliferation office of the US Department of Defense, and claim that despite its public position, the administration will eventually work behind the scenes to press Britain and Japan to reduce production of civil plutonium.

Meanwhile a high court ruling was expected in London yesterday (Wednesday) on whether the environmental group Greenpeace has the legal right to seek a judicial review of an earlier decision to let British Nuclear Fuels plc begin tests on the THORP plant. **Colin Macilwain**

Activists now urge caution on approval of new AIDS drug

Washington. In an apparent reversal of roles, scientists advising the Food and Drug Administration (FDA) have given the go-ahead for the use of the drug ddC on its own as a treatment for HIV — despite objections from AIDS activists that the potential value of the drug has yet to be demonstrated.

The FDA's Antiviral Drug Advisory Committee decided at a meeting in Rockville, Maryland, last week that ddC, which is produced by Hoffman-LaRoche, should be given FDA approval as a treatment for HIV patients who do not respond to AZT, or suffer side-effects from its use.

The decision was based on a trial conducted by the Community Program for Clinical Research on AIDS (CPCRA), which compared the drug's efficacy with that of the approved Bristol-Myers alternative, ddI.

But Gregg Gonslaves of the Treatment Action Group (TAG) in New York — one of several groups that opposed the application — told the meeting that approval of ddC could "open the floodgates for drugs bearing only the merest hint of clinical usefulness".

The panel's decision, and the objections of the activist groups, could mark a turning point in the politics of AIDS drug approval. In the past, many powerful activist groups have pushed for the early arrival of drugs onto the market, however tentative the evidence of their effectiveness, while the scientific establishment fought for restraint.

But faced with a number of disappointing trials, such as the European Concorde trial of the AIDS drug AZT (see *Nature* **362**, 483; 1993), groups such as TAG and the California-based Project Inform now want the FDA and the drug companies to do more to prove the efficacy of a drug before new treatments go on the market.

Deborah Cotton, a clinical researcher at Massachusetts General Hospital in Boston and an influential figure on the 13-strong committee, denies that the meeting's outcome means that divisions between scientists and activists are sharpening. "We are both trying to be pragmatic", she says.

The CPCRA study showed that patients who took ddC tended to survive longer than those taking ddI. "That probably was the key study that influenced the decision," says

David Feigal, director of the FDA's antiviral drugs division.

But AIDS activists at the meeting argued that the trial in fact showed broadly similar results for the two drugs. And they say that, despite the fact that ddI has already been given FDA approval, there is still insufficient evidence of the efficacy of either drug.

Cotton, who voted for the approval of ddC, says that she understands the activists' point of view. "But I felt the sponsor had demonstrated ddC's equivalence to ddI. While you may question whether either therapy is useful, I felt the trial design had been agreed to, and the company had done what it said it would do."

While giving their approval to the use of ddC on its own, the committee also advised the withdrawal of an accelerated approval process initiated last year for its use in combination with AZT. This was based on a trial by the National Institutes of Health's AIDS Clinical Trials Group which compared ddC, AZT and a combination of both.

The trial demonstrated possible benefits from combination therapy for some groups of patients. But, after hearing that it showed no overall improvement in survival time, the panel recommended that the accelerated approval be withdrawn.

The FDA's accelerated approval process involves special steps, such as the pre-vetting of all advertising, to ensure that the release of a drug onto the market is carefully controlled before its efficacy has been fully proved. Some members of the advisory committee are uneasy about the process.

The panel has now taken the opportunity to show that its approval can be withdrawn. In this case, they were able to reverse their previous positions painlessly because their approval of ddI on its own means that the drug will remain available to patients who are already taking it.

The FDA, however, is not obliged to accept the panel's advice. And it is likely to continue with the accelerated approval of combination therapy with AZT and ddC while the suppliers of the drugs complete a larger study of their efficacy.

But activists are growing increasingly concerned at the dilemmas they face. "We have arrived in hell," Gonslaves told last week's meeting. He can scarcely be accused of hyperbole. With no proof of efficacy for AZT, ddI or ddC, the question arises of whether new therapies and combinations should be tested against old, failed ones, or against neutral placebos. If the latter, patients and doctors must be found who are willing to forgo treatment with existing drugs in order to participate in the trials.

Colin Macilwain

France plans to raise science budget — or does it?

Paris. France's new conservative government plans to reduce the rate at which spending on science had been rising under its socialist predecessor. But, according to its budget for next year released in Paris last week, research funding will still rise faster next year than the planned 1.1 per cent

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François Fillon: promising more money.

overall growth in public spending. Particular increases will be targeted on medical science and industrial research.

French scientists are still trying to work out exactly how much better off they are going to be. François Fillon, the minister for higher education and research, says his proposed budget of FF51.6 billion (US\$9.3 billion) is 3.8 per cent higher than this year.

But Fillon was making the comparison with the revised budget for 1993. If the proposed 1994 budget is compared to the government's original request, the increase drops to less than 2 per cent. Fillon says that this comparison is invalid, however, because the former Socialist government proposed the original 1993 budget on the basis of "false growth projections".

To complicate matters further, Fillon's comparison is based on combining the costs of research overheads and actual cash payments; previous calculations on the size of the science budget were based on combining overheads and programme authorizations. If conventional comparisons are made, the proposed 1994 budget is only 1.2 per cent higher than the revised 1993 budget, and 2 per cent less than the original.

Even using Fillon's figures, however, French research organizations will have to live next year with budget increases only slightly higher than the anticipated 2.2 per cent rate of inflation. The budget of the Centre National de la Recherche Scientifique (CNRS) will grow by 2.8 per cent to FF12.4 billion; that of the medical research agency INSERM by 2.4 per cent to FF2.3 billion; and the agricultural research organization INRA by 2.2 per cent to FF3.1 billion.

In contrast, the French space agency CNES and the marine research agency IFREMER received proposed increases of 7.1 per cent (to FF8.7 billion) and 3.7 per cent (to FF0.93 billion). **Declan Butler**

Correction: LHC costs

The Large Hadron Collider proposed for the European Laboratory for Particle Physics (CERN) in Geneva is predicted to cost about SFr2 billion (less than £1 billion) and not £7 billion (*Nature* **365**, 96; 9 September 1993). This compares with current estimates of \$11 billion for the cost of the US Superconducting Super Collider.

Yeltsin raises pay for Russian scientists ...

Moscow. The Russian President, Boris Yeltsin, keen to stem the growing brain drain of scientific talent out of the country — and also to strengthen his political support in his bitter power struggle with parliament — has approved substantial salary increases for the country's leading scientists.

As from 1 November, full members of the Russian Academy of Science will receive a stipend of 150,000 rubles (about US\$1,100) a month on top of their regular academic salaries. Corresponding members of the academy will receive a stipend of half this value, namely 75,000 rubles a month.

These figures compare to the current monthly stipends of 10,000 rubles for full members and 5,000 rubles for corresponding members. There will also be increases for qualified researchers and for postgraduate students. Each of these will now be paid an additional stipend equal to half their salaries, and worth about 60,000 and 30,000 rubles respectively.

In addition, Yeltsin also announced that, as from next January, the government will select 5,000 "outstanding Russian scientists" who will be paid monthly stipends of 75,000 rubles each, in addition to their regular salaries. Further stipends of 50,000 rubles will be awarded to a thousand "promising young researchers".

These salary increases to academy members and other scientists have been approved by Yeltsin in a decree issued two weeks ago at the suggestion of the president of the academy, Yuri Osipov, a close colleague of the Russian president since his days in Sverdlovsk.

The decision to award monthly stipends to individual scientists is widely seen as Yeltsin's response to the proposal that the government should establish a system of "state professors". The proposal was put forward by Vladimir Zakharov, director of the Landau Institute of Theoretical Physics and a prominent member of the academy. Its aim had been to make the status and stipends sufficiently attractive to persuade leading scientists to stay in Russia and thus to curtail the exodus of its scientific élite.

The need for such an initiative has increased as a result of the creation of bodies such as the International Science Foundation, established by the Hungarian-born financier George Soros. Because of their charitable status, all of these foundations have decided to restrict their help to those who are in most financial need.

In contrast, the country's scientific élite, most of whom are still able to enjoy a relatively satisfactory standard of living as a result of their contacts with the West, have been largely neglected by such foundations. As a result, many leading Russian scientists feel increasingly deprived of the

opportunity to work in their own country.

Zakharov and others argued that it was up to the state to correct this situation by creating a system such as that of "state professors", and that those awarded such a title be given a relatively generous stipend of about 120,000 rubles a month.

But the proposal has been opposed by the presidium of the academy, apparently worried that a new system of state professors would challenge its own authority. The opposition from the academy establishment had, until now, been one factor holding back any response from the Ministry of Science.

But the new decree implies that the idea of state professors has finally obtained a legal backing — even if in a modified form — as a result of support from its strongest adversary.

According to the academic secretary and official spokesman of the academy, Igor Makarov, the scientific body is not opposed to the creation of state professors as such. And Andrei Gonchar, the academy's vice-president, said at a press conference in Mos-

cow that the new stipends should not be seen as an alternative to the idea of state professors as proposed by Zakharov.

But some observers are sceptical of this interpretation, as it seems unlikely that the government would support the simultaneous existence of 5,000 "outstanding scientists" and 5,000 "state professors", both supposed to enjoy the same scientific status but living on substantially different rates of pay.

At the current rate of inflation, the proposed stipends will have lost much of their value by January, and they are in any case only slightly higher than the official poverty level in cities such as Moscow and St Petersburg. It is therefore unlikely that, even though it will somewhat improve the financial situation of the impoverished Russian scientists, such an incentive will induce any truly outstanding Russian scientists to abandon the idea of emigration.

The decree appears to be linked to the recent instruction from Yeltsin to Osipov and the Minister of Science, Boris Saltykov, that they should present him within a month with suggestions on how the funding of science in Russia could be improved.

The main regret in the scientific community is that the new moves will not do enough to help the scientific élite. But more help is likely to come in a new decree from Yeltsin in response to a request from Osipov for more money for scientific equipment and laboratory supplies. **Vladimir Pokrovsky**

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Boris Yeltsin:
wooing scientists.

... but OECD says it has too many

Moscow. Russia's scientific community is three times larger than the country can currently afford to support, and should therefore take steps to reduce this number, according to the preliminary report of an expert panel set up by the Organization for Economic Cooperation and Development (OECD).

Based on an evaluation carried out at the beginning of this year, the OECD experts concluded that Russia is able to support at most 300,000 scientists and engineers. The report points out that so far between 200,000 and 300,000 posts have been cut, and that further large cuts lie ahead.

The OECD evaluation was carried out with the support of Russia's Minister of Science, Boris Saltykov. The group toured most of Russia's main scientific establishments, and prepared a report containing an analysis of the current situation and recommendations for action.

The report, which was discussed at a closed meeting with Russian officials in Moscow last week, gives high marks to the various steps taken by the Ministry of Science for the recovery of Russian science, such as the establishment of the Russian

Foundation of Basic Research and the creation of a network of scientific centres.

Specific recommendations included the need to create a state-level interdepartmental coordinating organization, to address the "brain drain", and to make the institutional changes that are necessary to shift the emphasis of the research community from the military-directed science to its civilian applications.

According to Saltykov, most of these recommendations "are in line with the decisions on which the Ministry of Science bases its policies". But he was less happy about the remarks that Russia has too many scientists — even though he has expressed similar opinions in the past — arguing that this was "a political question".

Saltykov, along with most of the scientific community, believes that it would be impossible to make drastic cuts at this time, partly because of the social dislocation they could cause in the scientific community.

The final report of the expert group's evaluation is expected to be completed in a few months, but it is not likely to differ substantially from the present version. **V.P.**

Japan promises more money for university research

Tokyo. Japan's Ministry of Education, Science and Culture (MESC) has asked for a 14 per cent increase in the money it has to spend on competitive research grants for university researchers in its budget request for the fiscal year starting on 1 April 1994.

If granted — as seems likely — the request will bring some welcome relief to Japan's cash-starved university researchers.

It also puts the ministry on target for achieving the goal set last year by its principal advisory council of almost doubling its research grants budget to ¥100 billion (about US\$1 billion) over the next few years (see *Nature* 358, 359; 1992).

The budget request, for a total of ¥83.9 billion, was submitted earlier this month to the Ministry of Finance. It is likely to be trimmed back by the ministry before being given final approval by the cabinet at the end of December. But only minor changes are expected.

A substantial increase has been requested in the plan to upgrade the university computer networks which will plugged into a new national "backbone" network. The local networks form part of a strategy that will result in a dramatic improvement in computer communications both within and between universities and national research institutes over the next year (see *Nature* 365, 7; 1993).

Funding for the overall project is also being sought by the Science and Technology Agency (STA) and the Ministry of International Trade and Industry. The backbone network, called SINET, runs from Japan's northern island of Hokkaido to the

southern island of Kyushu through the main island of Honshu.

Just over ¥30 billion — a 13.6 per cent increase — is being requested by the education ministry to upgrade the computer networks linking the universities. The new funding will increase the capacity of the network by a factor of more than ten, from 512 kilobits per second to 6 megabits per second. Branch lines will be increased from the relatively slow speed of 9.6 kilobits per second to 512 kilobits per second.

The whole network will be linked to the 6-megabit inter-ministry national backbone network planned by the STA, and the university network will also have two 2-megabit links to computer networks overseas.

The budget proposals also include MESC's first funding for its next major project in high-energy physics. The ministry is asking for ¥2 billion to begin construction of the B-meson factory to be built at the National Laboratory of High Energy Physics (KEK) in Tsukuba.

Japan's high-energy physicists have agreed that the B-meson factory should be their next major project. There had been fears that the Ministry of Finance would not be prepared to provide the financing if Japan is also required to contribute to the construction of the US Superconducting Super Collider (SSC).

But with the SSC struggling to survive in the US Congress, pressure on Japan to contribute has eased, allowing for the plans to build the B-meson factory to move ahead. The factory, which will be built within the ring of the existing TRISTAN electron-positron collider at KEK, should be operational within five years of the start of construction next year.

Japanese university researchers have been complaining for years about the small size of the ministry's budget for research grants. Yasutomi Nishizuka, for example, a leading molecular biologist at Kobe University, points out that the entire grant budget — which stands at ¥73.6 billion this year — is about the same as the budget of Johns Hopkins University in the United States.

The government budget for such grants is now rising rapidly. In contrast, there has been a dramatic slowing down in the expansion of funding from private industry to support

MESC's BUDGET REQUEST FOR SCIENCE IN 1994

	billions ¥	% change from 1993
Grants-in-aid of research	83.9	+ 14.0
Government/industry research	15.3	+ 0.5
Donations from industry	52.1	+ 4.0
Computer networks	30.3	+ 13.6
Nuclear fusion	13.2	+ 2.9
Accelerator physics (TRISTAN and B factory)	13.0	+ 6.9
Space science	21.5	+ 4.3
Astronomy (Hawaiian telescope)	4.8	+ 19.3
Earthquake and volcanic eruption prediction	2.7	+ 11.3
Antarctic research	3.8	+ 8.6
Japan Society for Promotion of Science (JSPS)	11.0	+ 22.2

university researchers.

By the end of the 1980s, following a period of rapid economic growth, the total of such research funds almost came to match those provided by the government. But as a result of the recession that has gripped Japan over the past two years, the ministry predicts that grants from industry will rise by only four per cent next year.

David Swinbanks

Senate seeks to cut NASA's search for "Green Martians"

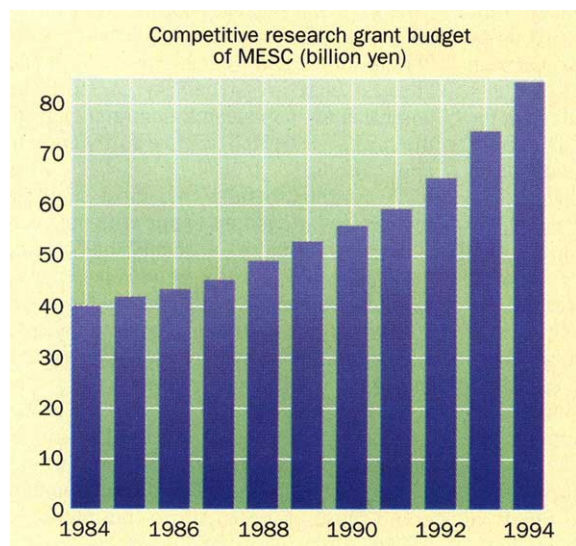
Washington. The US Senate appears to have delivered a fatal blow to a bid by the National Aeronautics and Space Administration (NASA) to mount a search for life in outer space.

NASA had tried to overcome congressional scepticism to its proposal by renaming its planned Search for Extraterrestrial Intelligence (SETI) programme as the High Resolution Microwave Study. John Rummel, chief scientist of the 10-year \$104-million programme, insists that it would involve good science, and points out that it has received repeated support from independent panels of the National Academy of Sciences and elsewhere (see *Nature* 364, 750; 1993).

But in moving an amendment to kill the coming year's funding for the project, Senator Richard Bryan (Democrat, Nevada), accused NASA of defying an earlier congressional instruction to cut SETI. The Senate backed the amendment by 77 to 23.

In theory, SETI could still be saved when a joint committee of the House of Representatives and Senate meets this week to finalize the budget. But the margin of the Senate vote makes this unlikely. "The Green Martian chase may finally come to an end," predicts Bryan. "This is a low priority and should be put on the shelf."

Colin Macilwain



Research grants for university scientists are on course to double within a few years.

Superphénix to become a plutonium 'furnace'

Paris. France is to reorientate its fast-breeder reactor programme — the last in Europe — towards burning-up plutonium generated from waste and dismantled weapons.

According to Jacques Bouchard, director of nuclear reactors at the French Atomic Energy Commission (CEA), the move has been prompted by the difficulty of justifying new fast-breeders when few conventional reactors are being built. The slowdown has also postponed the predicted uranium shortage that would have created a need for reactors that breed their own fuel, he says.

Similar considerations have already caused the United Kingdom and Germany to withdraw from the European Fast Breeder (EFR) programme late last year (see *Nature* **360**, 93 & **360**, 703; 1992). But CEA is convinced that it can persuade the government and utility companies to back the use of fast-breeders to transmute plutonium into elements with double-figure half-lives.

Waste from nuclear reactors and dismantled weapons will generate around 180 tonnes of plutonium by 2000, causing both security and environmental concerns. Furthermore, France imposed a 15-year moratorium on deep-storage of waste in December 1991, and its management has therefore become a national priority.

CEA's new programme is called CAPRA (Consommation Accrue de Plutonium dans les Rapides). Its first phase, which will finish at the end of 1994, will assess the economics, safety and technical feasibility of a plutonium furnace. CEA will then decide whether to convert its 1,200-MW Superphénix fast-breeder reactor, at Crey-Malville near Lyon, into a prototype for a purpose-built furnace.

The government, however, must first agree to restart Superphénix, which it closed in 1987 following repeated leaks in the liquid-sodium cooling circuits. The deci-

sion is due to be taken next year. Saving Superphénix is CEA's immediate goal. Last year, Hubert Curien, the then research minister, proposed in a report to the prime minister that the reactor be turned into a furnace. The new government is said to be impressed by Curien's conclusions.

Furnaces share the same basic design as plutonium breeders: a core of plutonium/uranium fuel coupled with a liquid-sodium cooling system. A breeder reactor produces more plutonium than it uses, because the fast neutrons it produces transmute a blanket of nonfissile uranium surrounding its core into fissile plutonium-239. Leave out the blanket, and it consumes plutonium.

CEA plans to equip Superphénix with cores lacking the uranium blanket in 1995 and 1999. Research will then centre on raising by a factor of at least three the estimated maximum rate at which fast-breeders now consume plutonium (25 kg per billion KW of electricity). A reactor of 1,200–1,500 MW could then burn 700 to 800 kg annually, says CEA.

Over the next 30 years, CEA proposes building one furnace for every five water-pressurized reactors in France, claiming that this would stabilize or even reduce plutonium stocks. Bouchard says CEA will also study how best to incinerate other actinide wastes, such as neptunium-237 and americium-241. He also hints that CEA might abandon oxide-based fuels in favour of metal fuels that are easier to process.

Meanwhile the US Congress is split over whether to support still a similar programme which would make use of the last remaining fast reactor in the United States — the Integral Fast Reactor in Idaho — for studies of plutonium recycling (see *Nature* **365**, 99; 1993).

The Senate has supported a proposal from the Department of Energy to keep the reactor open for a further five years in order to allow such experiments to be carried out. However, the House of Representatives has so far refused to vote any funds for such a programme.

Declan Butler

US plans to abandon Delaney Clause

Washington. The Clinton administration has proposed to abandon the 35-year-old Delaney Clause or "zero-risk standard" which governs the regulation of certain pesticide residues that concentrate in processed food or are added in food processing, and replace it with a uniform health-based safety standard that would apply equally to raw and processed foods and that would give no consideration to benefits. Under this new standard, pesticides would be judged as safe if there is "reasonable certainty of no harm" — the quantitative benchmark for which would be an upper risk estimate of one cancer case per million people over a lifetime.

The proposal is contained in a set of initiatives unveiled by the administration last week to improve the regulation of pesticides and raise public confidence in the food supply. The reform package, which would call for changes to both pesticide and food quality legislation, was presented to Congress by top officials from the Environmental Protection Agency (EPA), Food and Drug Administration and the Department of Agriculture.

"The need for change is urgent", said EPA Administrator, Carol M. Browner. Of the 600 pesticides that are now in use in the United States, Browner said that two-thirds have never been subjected to any health standard. One senior administration official described the current system of regulation as a "mishmash of competing and conflicting standards".

The administration's proposals have already come under heavy criticism from some environmental groups who see it as a softening of the current health standards regarding pesticide residues. Nevertheless, administration officials hope they can fashion a compromise bill with lawmakers that brings

together elements of two bills that have already been introduced into Congress.

One of the most controversial parts of the plan is the move to replace the Delaney Clause, which forbids the presence in food of any additive (including pesticide residues) that can be shown to cause cancer in laboratory animals. The administration now feels that, because of subsequent scientific developments, the Clause has become an anachronism, and that it should be replaced by a uniform standard of "negligible risk" applied to all pesticide residues in all foods, no matter whether the foods are marketed in raw or processed form.

At present, the Delaney Clause prohibits the approval of any pesticide that has been found to induce cancer in humans or animals if residues of the pesticide are found in processed foods above the level allowed in the raw agricultural commodity. Under these circumstances, approval is automatically denied without analysis of whether the level of risk is acceptable.

Diane Gershon

University head takes over at RIKEN

Tokyo. Akito Arima, the reform-minded former president of Tokyo University, is to replace Minoru Oda as president of the Institute of Physical and Chemical Research (RIKEN), one of Japan's foremost government research organizations, which is funded through the Science and Technology Agency.

Before taking up his position at RIKEN in 1988, Oda — who is retiring from the post this week — headed the Institute of Space and Astronautical Science (ISAS). He and Arima have been agents of change. Both have taken the unusual step of initiating

external reviews of their organizations; such initiatives are likely to continue after this week's transition at RIKEN.

Oda and Arima are old friends from Tokyo University's Institute for Nuclear Study, where they were both based in the 1950s. Oda will continue to serve as chairman of the board at ISAS and the National Astronomical Observatory. The space institute's director-general, Ryoji Akiba, backed by its board, is planning to launch an external review of the activities of ISAS in the near future.

David Swinbanks

Launch failure dents India's space plans

New Delhi. India's ambitious plans to enter the satellite launch business by 1995 received a major setback last week when its most powerful rocket failed in its maiden flight from the Sriharikota launch pad on the east coast, plunging into the Bay of Bengal shortly after take-off with its 850-kg cargo.

A preliminary report by a committee set up to analyse the failure says that the polar satellite launch vehicle (PSLV) seems to have been operating normally. It blamed the disaster on an "unexpected disturbance" during the separation of its second stage.

Whatever the cause, the failure is a blow to the Indian Space Research Organization (ISRO), already facing difficulties created by the imposition of technology embargoes

by the United States, and Russia's decision to back out of a contract to supply cryogenic technology needed for ISRO's geostationary satellite launch vehicle (GSLV).

A successful PSLV launch would have boosted the morale of the staff of both the ISRO and 150 companies who have been working on the PSLV project since 1983. U. R. Rao, the head of ISRO, says that the next PSLV will be ready for testing in 12 months, and that the launch vehicle will become available for commercial launch after three successful flights.

According to Rao, the PSLV flight was not a total failure because it demonstrated the effectiveness of two technologies being used by ISRO for the first time: a liquid

engine for main propulsion, and the world's third largest 128-tonne solid motor. As these pieces of equipment are also used in GSLV, ISRO feels reassured about its design.

With only three successful launches (out of seven) to its credit in 13 years, ISRO took a calculated risk in putting its new model on the launch pad before its two earlier models — the SLV-3, which can lift the equivalent of two suitcases, and the ASLV, which can carry 100 kg — are fully operational.

Observers say that ISRO was in a hurry to introduce the PSLV in order to gain quick access to the international launch market, which is already getting crowded with the entry of China, Japan and Russia.

Designed to place 1-tonne satellites in low Earth orbit, the 275-tonne PSLV is ideal for launching remote sensing and scientific satellites, says ISRO. The space organization spent \$140 million on the PSLV project over the past 12 years. But it says it can produce individual launchers at \$15 million each, offering a launch service at half the cost of those of Western countries.

India, a recent entry in the space business, formed a company called Antrix corporation last year. It has so far secured orders worth US\$300,000 from INMARSAT, a London-based satellite maritime communications organization. And ISRO is bidding for the contract to launch some of the 66 satellites that will make up Motorola's project for global communication with hand-held terminals, known as Iridium.

Antrix has already received several enquiries about Indian-built satellites. "This is because we make them cheaper", says Rao, who said Antrix can supply a geostationary communication satellite for \$24 million against \$100 million for a similar product from Western countries. "With four of our satellites now working beautifully in space, ISRO's credibility in satellite technology has gone up."

K.S. Jayaraman

Academies urge population control

London. The science academies of most of the world's leading industrialized and developing nations — including the United States, Russia, China and India — are planning to issue a joint statement next month on the need for governments to do more to control the growth of their populations.

The final text of the statement is due to be agreed at a five-day meeting in New Delhi, India. It is expected to state that population control is essential for any country wanting to raise its standard of living while preserving its environment, and to endorse a variety of measures, including in particular the promotion of both sex education and birth control techniques, to achieve this goal.

This is the first time that such a joint action has been taken, and the statement will be primarily addressed to the 1994 United Nations Conference on Population and Development. One proposal to be discussed in New Delhi is the creation of a standing committee to provide scientific advice to participants at the conference, similar to that established for last year's Earth Summit in Rio de Janeiro by the Intergovernmental Panel on Climate Change.

But the organizers of the meeting, described as a "science summit", also say that they hope the joint statement will be used by academies of science at a local level, particularly in the developing countries, to persuade governments to increase their efforts to reduce the growth of population.

The joint statement follows two separate initiatives, one a similar statement issued last year by the US National Academy of Sciences and Britain's Royal Society, and the other a meeting on population organized by the Royal Swedish Academy of Sciences. Both in turn were stimulated by a belief that the Rio meeting neglected population problems in addressing environmental issues.

"The meeting did nothing about the prob-

lem of population growth, despite all the talk about carbon dioxide and global warming", says Sir Francis Graham-Smith, physical secretary of the Royal Society. "We now have a worldwide network of science academies saying something needs to be done."

Replying to the charge that the meeting was neglecting the many social and economic pressures which also lead to the destruction of the environment, Graham-Smith said that the academies were focusing on the population issue "because that is the one side of this complex question which has been dodged in the past".

Among those attending the meeting will be delegates from the Vatican Academy of Sciences. But the academy is not expected to sign the final declaration. Graham-Smith acknowledges that the Vatican's opposition to birth control — expected to be repeated in a new encyclical shortly to be issued by the Pope — "would make it difficult for them to subscribe to any statement which comes out of the meeting."

David Dickson

London. Sir Mark Richmond (right) is to step down at the end of the year as full-time chairman of Britain's Science and Engineering Research Council to become director of the UK research division of the pharmaceutical company Glaxo. He will also co-ordinate the company's international research.

Announcing his decision last week, Sir Mark said that he had decided not to put his name forward as a candidate for the new post of Director-General of Research Councils, a position whose creation was announced in the recent white paper (policy document) on the organization of science.

He will, however, remain as part-time chairman of the SERC up to April 1 1994. This is the date on which the council is due to be split into

various parts, the biggest being the Engineering and Physical Sciences Research Council and the Particle Physics and Astronomy Research Council.

At Glaxo, he will take over from Richard Sykes — recently appointed chief executive — as head of the biggest private sector research and development programme in Britain. As such, Sir Mark is likely to continue to play a central role in science policy debates at government level.



Scientists protest at 'clean' appointments

Munich. Scientists at southern Italy's largest cancer centre, the Pascale National Cancer Institute in Naples, are planning a strike this week in protest at the shortcomings of a new administration imposed on them by Mariapia Garavaglia, health minister in Italy's interim anti-corruption government.

In particular, the scientists claim that the new administrative director, Giovanni Forte, lacks the scientific or administrative experience required to run the institute effectively.

Their protests are being backed by Enzo Mattina, the local representative in the European Parliament, who has called for Garavaglia's resignation on the grounds that Forte's appointment was the result of political favouritism rather than merit or skill.

The dispute at the institute follows charges against the previous administrators of the institute, accused of the now familiar crime of *tangentopoli* — taking cuts on contracts for the purchase of new equipment.

Suspected of close links with the former health minister and Christian Democrat Francesco DeLorenzo — now charged with operating a ring of corruption centred on the ministry (see *Nature* 364, 663; 1993) — the institute's administration was dismissed in its entirety in July by Garavaglia.

But Mattina claims that Forte's subsequent appointment was strongly influenced

by his own strong links with Italy's interior minister Nicola Mancino. The two come from the same small town in the mountains of the Campania region, of which Naples is capital. And Garavaglia and Mancino are in the same faction of the Christian Democrat party, implying a close working relationship between them.

The Pascale institute is the region's central cancer clinic, with 400 beds and at least 6,000 outpatients at any time. Its research facilities house 50 basic research scientists and 80 clinical research scientists.

Scientists at the institute have welcomed the government's anti-corruption drive. But since the arrival of Forte, they claim that the institute has not been functioning properly. For example, they say, requests for laboratory supplies are not being passed on by the administration. According to Giuseppe Castello, a clinical immunologist, grants from the European Communities have been received but cannot be spent.

Two months ago, the ministry of health, which funds the institute's research facilities, promised to double the number of basic scientists. But the promise has yet to be met, as the administration has not allowed the new positions to be advertised.

On the clinical side, requests for new supplies of drugs have been held up for so

long that stocks were expected to last only until the end of October. After an emergency meeting in Rome last week between Garavaglia, Forte and Marcos Salvatori, director of the Pascale Institute, a way has been found of buying in new stocks.

But routine cancer preventive measures, such as cervical smears, may still have to be suspended. Garavaglia has asked scientists at the institute for patience, claiming that the extent of change needed in Italy has slowed down the process of reform.

Part of the problem is that the ministry has responsibility only for the research side of the institute. In contrast, the clinical side is funded by the local authorities of the Campania region. And communication between the two has broken down. But according to staff at the institute, the main problem is Forte's lack of personal experience, and the difficulties that this has caused for the administrative staff working under him.

Scientists at the Pascale Institute have also found it frustrating that they have to negotiate with staff within the ministry who have not changed since DeLorenzo's demise. Government officials have been unable to comment either on the scientists' complaint of mismanagement, or on Mattina's charges of political favouritism.

Alison Abbott

Brain institute falls victim to war in ex-Yugoslavia

Zagreb. The construction of what was to have been the first large-scale science project in Croatia — the Croatian Institute for Brain Research (CIBR) in Zagreb — has been temporarily abandoned because the country's science budget has been exhausted by the war in ex-Yugoslavia.

The institute is the brainchild of Ivica Kostović, dean of the medical faculty at the University of Zagreb. It had been promised \$2 million by the Croatian Ministry of Science when the ministry was set up in 1990, and presented as the country's contribution to the European Decade of the Brain (see *Nature* 359, 200; 1992).

But all government support has now been withdrawn, and the skeleton of the 7,500 square metre building, originally planned to be opened in 1991 with 170 scientists, stands abandoned.

Construction work is unlikely to begin again in the near future. Zdenko Kovac, Croatia's deputy science minister, says that the sixfold decline in the science budget over the past three years means that the institute will have to seek support from private sources, at least until the Croatian economy picks up. But requests for endowments have so far been unsuccessful.

The CIBR's scientific programme, drawn



Building work at Zagreb's Institute for Brain Research is at a standstill.

up in 1990 by Croatian neuroscientists working both in the country and abroad, was to have focused on the neurobiological aspects of the development and ageing of the human brain, in particular the evolution of Alzheimer's disease and other degenerative disorders. The institute was to have housed the medical school's extensive collection of neurological samples taken from embryos, as well as its brain bank.

An international scientific advisory board, which included many expatriate Croatians now working in the United States, was set up in 1990 to evaluate all scientific proposals.

Unusually for Croatia, the institute's planned board of trustees was to have been made up of both foreign scientists and representatives of local governmental bodies.

The shattering of hopes for modern facilities has been devastating for those who had been planning to work there. "When the institute is finally set up there may be no scientists left to work in it", says Kostović, director of CIBR's scientific programme. "We are finding it hard to prevent young scientists from going abroad."

Kostović, who has continued to produce a steady flow of scientific publications despite the war, remains optimistic about the institute's prospects. He still hopes that one floor will be leased to companies wanting modern research facilities with access both to preclinical research in the institute and to Zagreb's neighbouring clinics. Kostović believes such sponsors can be found, and predicts a new opening date of May 1995 for the institute.

But Heiko Braak, a member of the advisory board from the Goethe University in Frankfurt, feels the optimism to be misplaced. Braak says that he has long been ready to provide antibodies for CIBR's research, but now thinks it unlikely that they will be asked for.

Robert Unterhuber

Whose research is it?

SIR — The letter by Alan Mackay (*Nature* 362, 489; 1993) on the handing over of copyrights to commercial interests points to a similar and perhaps more disturbing trend — that of effectively giving away the rights in one's research to pharmaceutical companies. Commercial companies are often the only source of compounds required to conduct investigations of physiological and pharmacological phenomena. They are usually willing to provide a 'free sample' of the compound(s), but only on condition that a restrictive agreement is signed, either by the researcher or by the head of his or her institute.

By these means, the investigator effectively transfers his or her rights and ownership of the research carried out to the company, and subjects his or her self to virtually the same conditions as the company's employees. Here are some examples of the wording used by pharmaceuticals companies in such contracts:

- Any discovery or invention (whether patentable or not) arising as a result of the studies with the compound(s) shall belong to XXX.

- All test results shall be submitted to XXX prior to informing any third party either verbally or in writing and they may be used further by XXX without restriction.

- At least 30 days prior to making any submission for publication or other public disclosure, I will provide XXX with a copy thereof for review and comment by XXX.

- Any proposal for omissions or modifications [to a manuscript] by XXX shall be respected.

In Denmark, the law prevents university employees from entering into a contract that would "... agree limitations to the researcher's right to publish results". Agreements with third parties of the type I have described are allowed only when the external party funds the research in its entirety. And, even then, "The rights to research which results from an agreement of collaboration belong to the researcher". I imagine that similar conditions apply in most countries, so was surprised when representatives of two companies told me that most investigators have no qualms about signing the contracts.

This situation is clearly unsatisfactory. I believe a two-tier system of agreements with the companies would be more appropriate, as follows:

- If a compound is to be used on the basis of a pharmacological property already documented for that compound, the investigator should inform the company of the purpose for which it will be used, and should declare that he will so limit its use, which should then be unrestricted.

- The investigation of novel compounds,

which requires absolute secrecy, that collaboration with independent investigators should be financed entirely by the company, which will then own the exclusive rights to the research.

D. C. Lambert

*Institute of Physiology,
University of Aarhus,
DK-8000 Aarhus C, Denmark*

Not so easy

SIR — You reported earlier this year on the setting up of the German-American Academic Council to support projects and exchanges in science (*Nature* 362, 779; 1993).

I came to the United States six years ago, newly graduated and full of optimism and energy, but I soon became disillusioned. Although my German diploma was officially evaluated as being equivalent to a master's degree, I could not find employment. When I considered working for a PhD, I was told that I would have to take certain undergraduate courses. I eventually got a job because my immediate supervisor is a European, but I was told that the institute does not recognize my degree. Many employers do not recognize foreign degrees below PhD level.

Now that I have been given the opportunity, I feel that I am making a valuable contribution to research. But I feel bruised and disillusioned, and when an opportunity arises I shall return to my own country, where my education counts for something.

Ute W. Sherman

*UTM.D. Anderson Cancer Center,
Science Park—Research Division,
Smithville, Texas 78745, USA*

Singular events

SIR — I am organizing a new publication called *Journal of Failures to Replicate*. Individually, I am sure that many of us have experiences of seriously doubting the replicability of reported studies that ultimately find their way into the textbooks. More importantly, on a philosophical level, there are serious implications within our disciplines for the issue of "when does a finding become an established fact?"

I am keen to hear from anyone who is interested in contributing articles or acting as an academic referee. Naturally, contributions should concern fundamental issues within their relative domains.

Derek Scott

*Department of Psychology,
University of Strathclyde,
Turnbull Building,
155 George Street, Glasgow G1 1RD, UK*

Neural net effect

SIR — In his recent review of Daniel Crevier's book *The Tumultuous History of the Search for Artificial Intelligence* (*Nature* 364, 199; 1993), Igor Aleksander writes:

"Recent work in connectionism has shown that distributing knowledge over interacting elements (artificial neurons) could lead to a storage requirement that grows only with the logarithm of the number of facts that need to be stored. It seems likely that the brain would have evolved to use this neat trick."

Could he refer your readers to the evidence for this astonishing claim?

H. Christopher Longuet-Higgins

*University of Sussex,
Centre for Research
on Perception and Cognition,
Laboratory of Experimental Psychology,
Brighton BN1 9QG, UK*

Rise and fall

SIR — Regarding the argument¹ from the unlikelihood of our being lucky enough to be at the beginning of human civilization and so predicting our species' demise, we are not a random sample. We are at the beginning, because only the early civilized people will not immediately spot this flaw in the argument!

A. J. Hardwick

*University of Cambridge,
Department of Physics,
Cavendish Laboratory,
Madingley Road, Cambridge CB3 0HE, UK*

SIR — In this note we shall use the Copernican principle¹ to estimate the life of Conservative government in Britain. Of course, we have to make the (dubious) assumptions that we are at no special position on the time axis and that there exists an appropriate uniform distribution. Then, using the resulting equations (1) and (2) from ref. 1, with $t_{past} = 14$ years, one finds that (at 50 per cent confidence level) current Conservative rule will continue for a period lying between 4.7 and 42 years. For the worried Conservative, one may say, with 95 per cent confidence, that the future period of their preferred mode of rule is not less than 4.3 months. Those less enthusiastic about the present state of political affairs may draw some comfort from the fact that, at the same 95 per cent confidence level, the current Conservative rule will end within the next 546 years.

P. T. Landsberg

J. N. Dewynne

C. P. Please

*Faculty of Mathematical Studies,
University of Southampton,
Southampton SO9 5NH, UK*

1. Gott III, J. R. *Nature* 363, 315–319 (1993).

Even the social sciences have laws

Who says that the social sciences are not quantitative? Even in a field supposed to be dominated by people's impulses to buy — that of marketing — there are striking regularities.

A MINOR brand usually has the worst of two worlds: compared with major brands, it has fewer buyers and they are usually less loyal. They buy the brand less often and they like it less. This phenomenon is called Double Jeopardy (DJ).

In marketing, the pattern is almost universal, much as the planets are always almost spherical. Anybody could have noticed it, but for a long time did not. Although the US sociologist William McPhee described Double Jeopardy 25 years ago, most marketing people have remained unaware of it. The research question is therefore not so much why DJ arises, but why the pattern been so often missed?

To illustrate the prevalence of DJ, suppose there are just two restaurants in town: A is widely known, B less well so. Suppose that the two restaurants are of equal merit in every respect to those who know both. What then happens if people in general are asked which is their favourite? People who know B as well as A will "split their vote", because both restaurants are of equal merit. But people who know only A will all vote for it. That is DJ in action.

Much the same happens when people have to choose between similar items differing significantly in popularity or market shares. Marketing people have documented the phenomenon thoroughly in the past 40 years in many countries and a great variety of circumstances: motor-cars, retail chains, grocery brands (in over 50 products), television and radio programmes, newspaper reading, comic strips, politicians and even consumers' attitudes to the items in question.

Experience has shown that the buying patterns of competing products may usually be represented by the Dirichlet distribution, of the form $Cp^{\alpha-1}q^{\beta-1}r^{\gamma-1}\dots$, in which $p, q, r, \dots$ represent consumer preferences (over a finite range, say $[0,1]$), $\alpha, \beta, \gamma, \dots$ are the corresponding market shares and the constant C is a function of $\alpha, \beta, \gamma, \dots$. This formulation successfully and rather precisely predicts many regularities, including DJ.

The model needs only one measure for each brand: its market share. Other market influences, such as differences in product formulation, advertising expenditure, price, availability or whatever, are not explicitly involved. They will have given brands their widely differing market shares, but they do not affect brand loyalty beyond that, either in the theory or in practice.

There are many irregularities in the marketing process that might be expected to override DJ, which is supposed to happen when competitive items are very similar.

But even competitive items differ somewhat, at least in name. Yet the facts show DJ is the rule, not the exception.

There is an extreme example in the London newspapers: *The Times* and the mass-appeal *The Sun* differ even to the naked eye (although the owner is the same). But there is still DJ; not only do fewer people read *The Times*, but they read it less often. Exceptions to the DJ rule are few and far between and have specific explanations.

DJ would be less remarkable if customers of a small brand had limited needs and satisfied them with that one brand. But the opposite is what happens. In general, buyers of brand X tend to buy other brands as well, and in total (over time) more often than they buy brand X itself. Consumers generally spread their choices across a portfolio of competitive items.

There are practical benefits of this knowledge. For example, managers who know about DJ can more accurately evaluate the intrinsic value of their brand. If they recognize that it is normal for a small brand to command the lesser loyalty, they will be better able to allocate marketing budgets, to set targets for new brand development and to interpret the results of market testing.

Thus for established brands, DJ confirms that the only way of doubling sales is to attract almost twice as many buyers to purchase the brand slightly more often, not to persuade all current buyers to purchase twice as often (which would require an upheaval of market structure.) Even the now fashionable "niche" brands, often designed especially to attract few but exceptionally loyal customers, suffer the same DJ effects as small brands: fewer buyers with relatively low loyalty. No striking exceptions have been reported.

Given the ubiquity of the phenomenon of DJ, it is not surprising that its effects have been discovered more than once. More than 20 years ago, it was first noted (with surprise) that different brands of a category of packaged goods commanded similar loyalty, measured by repeat-buying. Then a subpattern became apparent: such small variations in loyalty as there were systematically decreased with the market share of a brand. But that trend was then recognized to be just what McPhee had noted, explained, and named as Double Jeopardy a few years earlier when analysing radio announcers' popularity.

Remarkably, neither marketing academics nor practitioners have generally been aware of DJ in their markets. One reason is that people seldom expect there to be lawlike regularities in social science ("Is it a science?"), and therefore do not even look for

them. In this they are oddly aided and abetted by modern statistics, which concentrates almost exclusively on analysing single sets of data ("Is it significant?") instead of many sets of data ("Does it generalize?").

Another reason why the DJ phenomenon has often been overlooked is that statistical data are often unhelpfully communicated. The figure below gives data on aviation fuel contracts in Europe, with the brands listed in order of decreasing market share. It is plain that the companies supplying aviation fuel have very different market penetrations (percentage of airlines who are customers) and that, as market penetration decreases, so does the number of contracts per customer (a measure of loyalty).

None of the sophisticated statistical procedures of modern management science (regression, factor analysis, multidimensional scaling or whatever) are needed to see the pattern, which may nevertheless be hidden if the numbers are not rounded to two effective digits and, most importantly, if the companies are not ordered in a relevant way.

"Brand"	Percent airline customers	Contracts per customers
Shell	73 (72)	3.5 (3.7)
Minors	51 (60)	3.5 (3.1)
BP	44 (44)	2.5 (2.6)
Total	28 (31)	2.5 (2.3)
Mobil	28 (29)	2.3 (2.3)
Exxon	28 (27)	2.1 (2.3)
Chevron	19 (16)	1.7 (2.1)

DJ in airline fuel. Figures in bold are Dirichlet predictions.

Such low-level data communication techniques are seldom practised but sorely needed. They help to uncover patterns and to suggest whether these patterns generalize. Large companies (and universities) spend much time and money collecting data on their markets and worrying about it. But such data often conceal general or lawlike patterns that can be documented and established, leading to far better interpretation. Do university departments that receive fewer applications also have fewer of their offers taken up? That would be normal, another DJ effect. When belief in unguided market forces is as widespread as at present, a better understanding of how competitive markets and their lawlike patterns actually work would not be out of place. And how does Double Jeopardy affect you?

Andrew Ehrenberg

Andrew Ehrenberg is professor of marketing at the South Bank Business School, and visiting professor at the Stern School, New York University.

A question of conformation

Kimberly Carr

WHEN shown a videotape of a cow with bovine spongiform encephalopathy (BSE), inhabitants of the Fore region of Papua, New Guinea, responded instantaneously — their impression was that the animal was suffering from kuru, a neurodegenerative condition associated with ritual cannibalism. That reaction was perhaps predictable, for both diseases result in a characteristic, staggering gait. Both are also caused by prions, as are several human diseases, and these mysterious entities came under scrutiny at a meeting last week*.

Prion diseases have a prime claim on public attention. The epidemic of BSE in Britain continues (although it is said to be past its peak). And there have been reports from around the world of cases of Creutzfeldt–Jakob disease (CJD) in which the disease has been transmitted to people after their treatment with the contaminated products from human cadavers.

Kuru, scrapie, BSE and other transmissible spongiform encephalopathies are caused by infection with particles containing an abnormal form of the prion protein, which is normally encoded by a chromosomal gene. Familial Creutzfeldt–Jakob disease, on the other hand, like Gerstmann–Sträussler–Schenker syndrome and the most recent addition to the list, fatal familial insomnia, is due to a mutation in the chromosomal prion gene.

The onset and form of inherited CJD are affected by polymorphisms in the prion gene. The disease has an early onset in patients homozygous for a particular mutation at codon 129 of the gene (J. Collinge, St Mary's Hospital, London) and other polymorphisms have been reported in different populations. Japanese CJD patients have four amino-acid changes not seen in other populations (T. Kitamoto, Kyushu University), and Libyan Jews with CJD have a mutation at codon 200 which seems to lead to abnormal glycosylation of the cellular protein, possibly affecting its metabolism (R. Gabizon, Hadassah University Hospital, Jerusalem). In addition, susceptibility to kuru and iatrogenic CJD may be genetically determined (L. Goldfarb, National Institutes of Health, USA; J. Collinge).

But what does the wild-type form of the prion protein (PrP^c) do? Mice without PrP^c seem to be entirely normal, although they are completely resistant to prion infection (C. Weissmann, University of Zurich). In attempts to understand the role of the protein in uninfected animals, cellular proteins binding to PrP^c in the brain are being characterized. A 110-K

protein that seems to be associated with the membrane has been found, but so far its function is unknown (B. Oesch, University of Zurich). Another clue may be provided by studies involving overexpression of PrP^c in transgenic mice, which leads to a late-onset neuromyopathy (G. A. Carlson, McLaughlin Research Institute for Biomedical Sciences, Great Falls, Montana).

As to the way in which abnormal forms of the protein cause disease, it was suggested some time ago that the infectious prion particle (PrP^{Sc}) is simply an abnormal form of the cellular protein with an altered conformation, the interaction of which with cellular protein causes the cellular protein to adopt the disease-related conformation (S. Prusiner, University of California, San Francisco). It is well-established that the abnormal form of the protein is composed largely of β -sheet. But the conformation of the normal form was unclear until recently. Fourier transform infrared spectroscopy has now been used to show that the normal form is mainly α -helical, and attempts to re-create the conformational change from one form to the other *in vitro* are under way (M. A. Baldwin, University of California, San Francisco).

For those who still find it difficult to accept the 'protein-only' hypothesis, the search for a nucleic acid responsible for prion infectivity continues. Any treatment that destroys the β -sheet structure of PrP^{Sc} also destroys infectivity of the prion particle. But the possibility of a nucleic acid somehow trapped in the infectious particle cannot strictly be ruled out without carrying on the search to its logical conclusions. From studies using return refocusing gel electrophoresis and other methods, however, it seems that if there is a nucleic acid in the prion particle it must be very small (D. Riesner, Heinrich-Heine University, Dusseldorf).

If the abnormal form of the protein is indeed solely responsible for prion infectivity, then what accounts for the different properties of various 'strains' of prions? A number of studies point to post-translational, possibly cell-specific modifications of the protein (S. J. De Armond, University of California, San Francisco) leading to conformational differences. In addition, the binding of an endogenous glycosaminoglycan seems important for prion replication (B. Caughey, Rocky Mountain Laboratories, Montana).

In Britain, it is BSE that has been the greatest cause for public concern. Epidemiological studies indicated that cows can contract the disease from feed containing meat and bone meal from

sheep infected with scrapie; as a result, such feeding practices were banned in 1988. In 1989 there was a leap in the reported number of two-year-old cows with BSE, but this was probably because by 1987 feed contained meat and bone meal from BSE-infected cows, as well as from scrapie-infected sheep. This is a notable example of the 'species barrier': the first transmission of a prion to a new species shows lower infectivity and a longer incubation time than subsequent passages in that species. The good news is that the ban seems to be having the desired effect, and the number of new BSE cases is now falling (J. W. Wilesmith, Central Veterinary Laboratory, Weybridge, Surrey).

The big question, of course, is whether or not BSE can be transmitted to people who eat infected beef. So far, there is no evidence of transmissibility to humans, but BSE does seem to be responsible for spongiform encephalopathies in domestic cats and zoo animals, probably due to infected feed. Unlike other transmissible spongiform encephalopathies, BSE seems to have a strikingly dominant effect, maintaining its strain characteristics (such as neuroanatomical distribution and incubation time) even after passage in other species (M. E. Bruce, Institute for Animal Health, Edinburgh).

It would be premature to raise an alarm, but studies of the possible transmissibility of BSE to humans are clearly in order. As the effects of the abnormal prion protein are influenced by its homology to the cellular protein, studies in primates are not necessarily appropriate. Experiments are planned using mice transgenic for the human prion protein in order to assess their susceptibility to BSE (J. Collinge).

Evidence for the 'protein only' hypothesis continues to accrue. Transgenic mice carrying high copy numbers of a prion gene with the Gerstmann–Sträussler–Schenker mutation spontaneously develop symptoms mimicking the human disease, and recent experiments have shown that brain extracts from these mice contain infectious prions (S. Prusiner). These experiments would seem to rule out the effect of foreign nucleic acids, as no external source of the infectious agent is required. In News and Views over two years ago (*Nature* 349, 569–571; 1991) Charles Weissmann argued that such results would provide a case for the 'protein only' hypothesis that would be convincing beyond reasonable doubt. It remains to be seen if this mechanism by which an abnormal form of a protein induces the production of more abnormal forms from normal cellular proteins is valid only for the prion diseases, or if such a mechanism is involved in other diseases as well. □

Kimberly Carr is an assistant editor at Nature.

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Elusive diffusive liquids

N. W. Ashcroft

ESTABLISHING the phase diagram of elemental carbon¹ remains a challenge, mainly because thermal destruction of the common diamond and graphite phases is not thought to set in until around 4,500–5,000 K. If they form a diffusive liquid, it remains elusive; there is no clear picture of what the mobile statistical units might be (single atoms? transiently bonded clusters?). As we learn to fabricate larger units of carbon, and particularly the remarkably stable C_{60} , it is reasonable to ask whether these have corresponding fluid phases, and ones that may even set in at much lower temperatures. Hagen *et al.*, on page 425 of this issue², conclude that C_{60} will not possess a conventional liquid phase at all. But last month, Cheng *et al.*³ used the same information and reported the opposite conclusion.

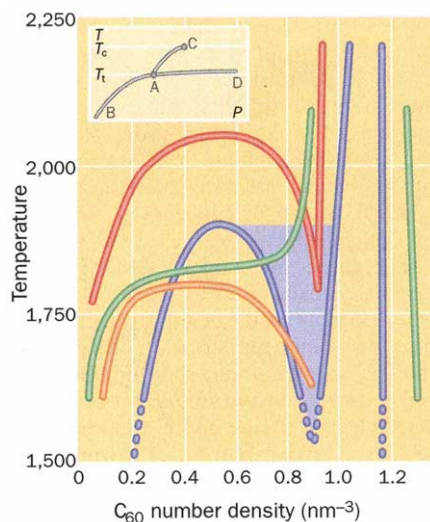
In a composite of the phase diagrams of both groups (see figure), there is certainly broad agreement between the calculations at the temperatures that seem relevant. Is the divergence in conclusions just a matter of definition, a different view of what constitutes a liquid?

The issue for C_{60} seems to go deeper, as simple arguments show. Dense fullerene is fairly incompressible, and its assumed pair potential is sharply repulsive at a separation of around 10 Å. The molecules are near-spherical, so a plausible starting viewpoint of dense fullerene is an assembly of identical rigid spheres, of diameter σ , with milder attractive interactions between them (which lead to the observed cohesion of about 1.6 eV per molecule). The simplest approximation to the statistical mechanics of this system is to omit for the moment the attractive forces, and to stabilize it merely by applying an external pressure, P . This quickly leads us to the well studied effective hard-sphere model of atoms and molecules⁴.

Some physical attributes are easy enough to establish. For example, if we proceed within classical physics, a state of maximum density can result when the system has no motional energy. This state is a regular crystal, with the molecules touching, and it is plainly non-diffusive. The arrangement is face-centred cubic. Apart from some orientational complexities, important in their own right, the low-temperature packing of C_{60} is exactly of this face-centred-cubic type.

The effects of temperature T are now easy to see. Imagine the ratio of lattice to sphere size is expanded just a little, and endow the system with some internal energy. As a system of rigid spheres cannot acquire any potential energy, the energy E must be entirely kinetic. The corresponding motion must therefore be

violently collisional (it is a system of utterly anharmonic 'collisional oscillators'), but for a small enough expansion no 'escape paths' will open that are large enough on average to let a given molecule wander from its average crystalline position. But if the expansion is permitted to increase systematically, escape paths will appear when the lattice constant rises



Comparison of the two proposed phase diagrams for C_{60} molecules. Temperature is in kelvin. Green: boundary between liquid and solid phases according to Hagen *et al.*². It lies above their line that would separate vapour and liquid (orange). Blue lines, from Cheng *et al.*, are of a more conventional form, indicating (as does their theoretical estimate; pink) the existence of all three phases including the liquid (shaded region). Inset: Equilibrium of phases of a simple substance in a (T, p) plane. Curve AC (ending in a critical point) separates vapour from liquid. AD separates solid from diffusive states. AB, the sublimation curve, separates solid from vapour. Pressures must be less than about 20 GPa to preserve the integrity of the C_{60} molecule⁷.

from its close-packed value, 1.414σ , to 1.62σ , at which point simulation studies confirm that the system undergoes a slight downward jump in density to a different state. Here, periodic order has been destroyed by a process we would call melting, and in the new phase the molecules are diffusive: in time all points in the sample are accessible to them. As the density of a simple solid changes little during melting (about 10 per cent for hard spheres) the motions must remain intensely collisional.

So we expect a striking change in phase of the packed C_{60} at some temperature. The wandering states are vastly different from their localized equivalents, and behind the argument is the powerful hand of entropy, S . As a problem in thermodyna-

mics, the preferred state of our system is always one of lowest Helmholtz energy, $E - TS$. Since the internal energy must be independent of state, the entropy of the effective hard-sphere model of C_{60} in the diffusive disordered phase is actually lower than that of the ordered crystal at the same density. Is C_{60} sufficiently odd in terms of its interactions that this old result for hard spheres will survive in part when we add the attractive forces to the model? It is precisely these attractive forces that give rise to distinct liquid and gas phases in real substances — they put the well known loop into the Van der Waals equation of state. It is certainly better to think of dense C_{60} as an assembly of 'sticky-hard-spheres', and we might also then expect the solid phase to change from a system of collisional oscillators to a system of possibly harmonic oscillators. Just how harmonic depends on the excitation energies, and for extremely steep short-range repulsions some aspects of the anharmonic character of hard spheres persist.

In the diffusive phase another fundamental physical change can take place. Previously all densities of this phase were open to the system, by appropriate adjustment of pressure and temperature. Now, in principle, a sharp division in behaviour can occur at a temperature T_c , characteristic of the overall interactions, and below which there are ranges of density that are excluded to the system as a single phase. Instead it breaks up into two phases, both still diffusive, and in equilibrium. One of these has a higher density, and this we conventionally call the liquid.

Given the attraction between C_{60} molecules, collapse to a high-density diffusive phase is certainly a possibility. But is the attraction sufficient? After all, in some quite simple systems sublimation is possible; in a given material it is only a question of where the triple point pressure is in relation to the chosen (usually atmospheric) pressure. The question being raised by Hagen *et al.*² is whether for C_{60} the attractions are such that no liquid phase can ever develop.

Both Hagen *et al.*² and Cheng *et al.*³ note that when viewed in terms of familiar scalable forms of potentials (Lennard-Jones is an example), the pair potential for C_{60} is anomalous. It is certainly deep (the well depth is about 0.3 eV, or about 3,200 K in equivalent temperature), but it seems to be very short-ranged⁵. Is the attraction actually a large perturbation on the packing problem? We can estimate this by using the standard first-order argument that leads to the Van der Waals equation. If the attraction falls off with separation with a power m significantly larger than 6, then for equal depths the perturbation will be smaller than normal Van der Waals attraction ($m=6$), by roughly a factor of $3/(m-3)$. So before a Van der Waals loop is generated, the

system might simply choose to lower its free energy by reverting to a solid.

In pursuit of these possibilities, both groups work with the same suggested form for the intermolecular potential⁵. Cheng *et al.* also receive guidance from parallel theoretical estimates which, incidentally, do predict the possibility of a restricted liquid region. But the simulation methods used are not identical, nor are the criteria invoked to locate the phase boundaries, so differences are inevitable. The most obvious is the persistence of the liquid phase in the results of Cheng *et al.*, whereas Hagen *et al.* find a solid-liquid phase boundary that lies a little above their liquid-vapour phase boundary. From this they conclude that C_{60} has no self-stabilized liquid phase.

A moot point for C_{60} is the assumption that its configurational energies are representable by spherically symmetric pairwise potentials alone. Apart from the assumption that rotational contributions to energies are common in all phases, the main issue is whether three-molecule and higher interactions are significant for molecules such as C_{60} with large linear polarizabilities. It turns out that the polarizability and density in dense C_{60} are together large enough that the condition for existence of an expansion of energies in potentials⁶ is close to being violated. It is common enough to argue that higher interactions can be approximately incorporated into effective pair potentials through the fitting procedures used to determine them. But the resulting state dependence has an uncertain effect on the delicate matter of determining a phase boundary.

When faced with a common element in a new guise, we sorely need calculations like these, for they can provide critical insights into questions fundamental to the structure of matter. Although they are studies of 'model C_{60} ', they represent an essential first step in assessing what phase structures are possible in an unprobed regime of the system. 'Real C_{60} ' will no doubt present further challenges. But in principle, similar suitably modified dynamical studies could help with the puzzle of why some fullerenes intercalated with metals (such as K_3C_{60}) are excellent superconductors and others (such as Na_3C_{60}) are not. □

N. W. Ashcroft is currently at the Laboratoire d'Etudes des Propriétés Electroniques des Solides, CNRS, Grenoble, France.

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A tale of two messengers

Michael J. Berridge

CELLS have two intracellular channels for regulating calcium release from internal stores¹; ryanodine receptors, first discovered in muscle but now known to exist in other cell types, and inositol trisphosphate ($InsP_3$) receptors. Although the messenger responsible for opening the latter was known to be $InsP_3$, an analogous physiological regulator for the ryanodine receptors was not known. Two recent papers^{2,3} provide mounting evidence that ryanodine receptors might be controlled by cyclic ADP ribose (cADPR) (Fig. 1), and interest in this putative second messenger is likely to grow following the report by Galione *et al.* on page 456 of this issue⁴ indicating that the level of cADPR in sea urchin eggs is regulated by cyclic GMP.

The new messenger, cADPR, was originally discovered in sea urchin eggs, where it mobilized calcium from an $InsP_3$ -insensitive calcium store⁵. The first hint that a ryanodine receptor might be its target emerged from studies on sea urchin eggs where caffeine and ryanodine abrogated the response to cADPR while leaving intact the calcium release induced by $InsP_3$ (ref. 2). More direct evidence has come from studies on cardiac ryanodine receptors in planar lipid bilayers where cADPR greatly increased the frequency of channel openings³. cADPR has no effect on the type 1 ryanodine receptor of skeletal muscle so its messenger function is restricted to the type 2 cardiac receptors³ and perhaps also to the type 3 receptor which has been found in certain cell types¹. Although these direct effects of cADPR seem to support a messenger role, a note of caution is required because unpublished observations circulating

within the calcium fraternity indicate that cADPR has no effect in certain cells. Perhaps the messenger function of cADPR is much more restricted than that of $InsP_3$?

Before cADPR can be accepted as a messenger, it will also be necessary to show that its level changes in response to specific stimuli. Following in the footsteps of studies of messengers such as cyclic AMP and $InsP_3$, there are teething problems in developing sensitive assays. A biological assay has been used to show that the level of cADPR changes in pancreatic β -cells in response to stimulation with glucose⁶. Using the precursor labelling protocol which proved so useful for measuring both cAMP and $InsP_3$, Galione *et al.*⁴ show that the formation of cADPR is enhanced by cGMP, which is known to trigger fertilization in sea urchin eggs. If activation by cGMP occurs in other cell types, it suggests a possible link with the local hormone nitric oxide (NO) (Fig. 1). It remains to be seen, however, whether the level of cADPR changes in response to conventional extracellular signals such as hormones and neurotransmitters. Nevertheless, this link to cGMP adds to the growing evidence that cADPR might be the messenger that is responsible for regulating certain types of ryanodine receptors, in the same way that $InsP_3$ controls the $InsP_3$ receptor.

These two messengers share many similarities. They are both formed through a single enzymatic step from well-known precursors. Of particular importance is the way they sensitize their target receptors to the stimulatory action of calcium. This co-agonist principle is well established for the $InsP_3$ receptor, which

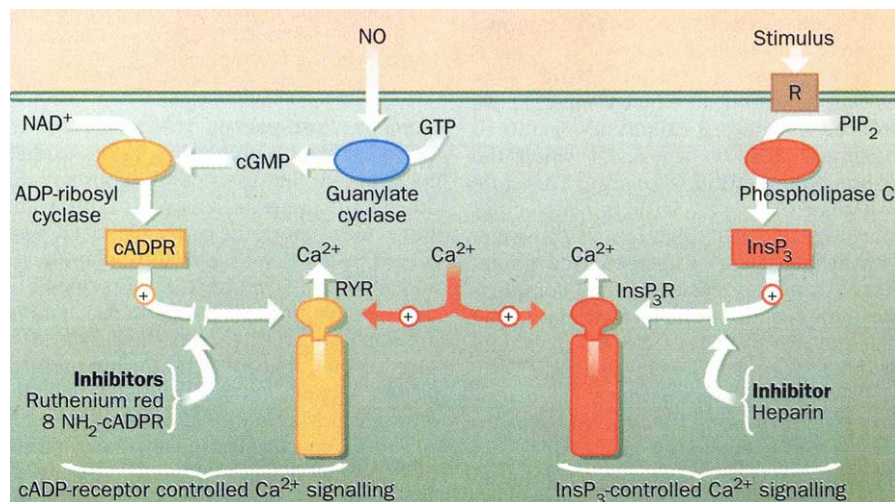


FIG. 1 Calcium-mobilizing second messengers. Two separate signalling pathways activate the ryanodine receptor (RyR) and the inositol trisphosphate receptor ($InsP_3R$) respectively. cADPR, cyclic ADP ribose; NO, nitric oxide; PIP_2 , phosphatidylinositol bisphosphate.

is controlled by both InsP_3 and Ca^{2+} . A similar phenomenon seems to exist for ryanodine receptors in that cADPR potentiates the process of calcium-induced calcium release⁷. This ability of InsP_3 and cADPR to sensitize their receptors to the stimulatory effect of calcium provides a plausible mechanism for the generation of both calcium oscillations and calcium waves¹.

As many cells have both ryanodine and InsP_3 receptors, we have to consider how the two messenger systems may interact to generate calcium signals. Both messengers contribute to the fertilization calcium wave in sea urchin eggs^{8,9}, and there is clear evidence of redundancy in this case. Inhibition of either pathway had no effect (the cADPR-sensitive pathway can be inhibited by either ruthenium red or 8-amino-cADPR (ref. 8), whereas heparin blocks the InsP_3 receptor^{8,9} (Fig. 1)); the fertilization wave was abolished only when both messengers were knocked out. What is surprising, however, is that when only one pathway was inhibited, there was little change in either the amplitude or the velocity of the calcium wave⁸. Some alteration in the parameters of the wave would be expected if both mechanisms were normally operative. It would seem, therefore, that only one mechanism is normally used while the other is held in reserve. Such a back-up mechanism based on cADPR is absent in amphibian and mammalian eggs, which depend solely on the InsP_3 pathway.

Apart from serving as a back-up for each other, these two messengers may interact in more subtle ways, especially where the stores they regulate are spatially segregated as in neurons. For example, the soma and dendrites of Purkinje cells have both messengers but only InsP_3 receptors are present in the dendritic spines. We know very little about what these receptors are doing in the central nervous system. But it is reasonable to speculate that they could function as 'coincidence detectors' which have the job of integrating separate neural inputs during the acquisition of memory.

Although the phenomenon of long-term depression in Purkinje cells is somewhat controversial, it does provide a good example of coincidence detection¹⁰. Synaptic transmission through the parallel fibre synapses, which innervate the spines, can be depressed for a prolonged period if they are fired within a short interval of activating the climbing fibres which end on the dendrites (Fig. 2). This example of neuroplasticity depends on an increase of calcium within the spines which could result from a collaborative interaction between these intracellular receptors.

First, the activation of climbing fibres initiates a calcium spike that spreads up the dendrites through the opening of

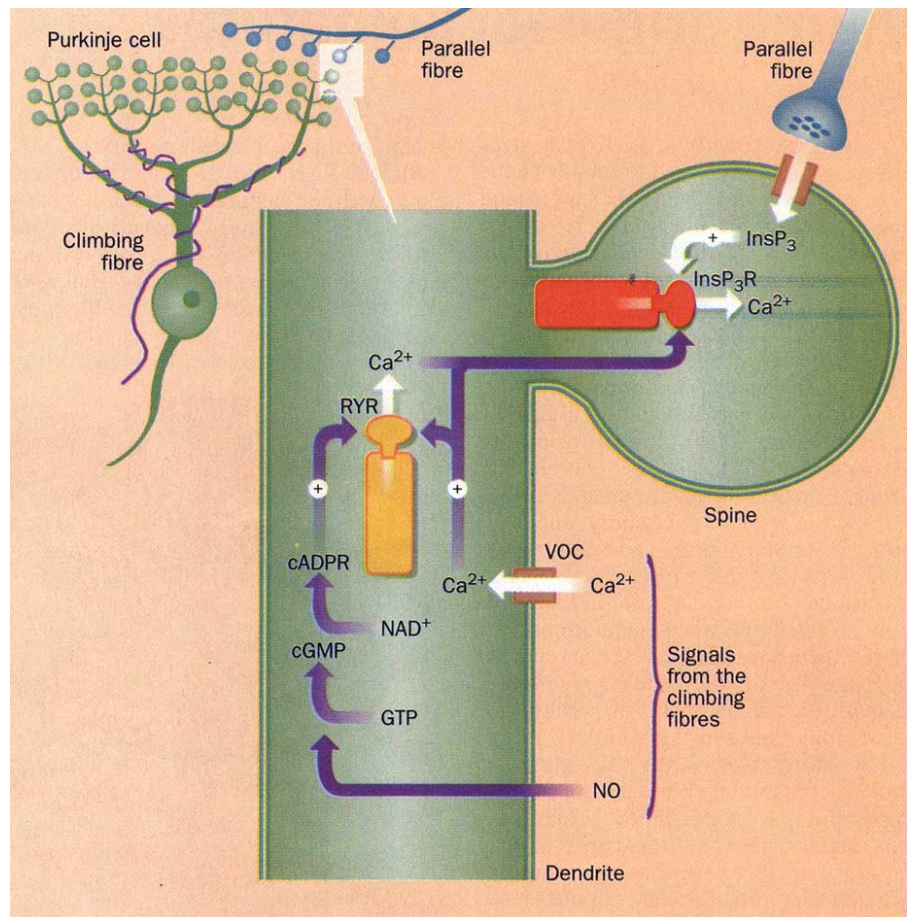


FIG. 2 Proposed function of calcium-mobilizing receptors as coincidence detectors in Purkinje cells. Ryanodine receptors (RYR) may use cADPR to amplify dendritic Ca^{2+} spikes resulting from the opening of voltage-operated channels (VOC). Spines have InsP_3 receptors (InsP_3R) which may integrate this climbing-fibre Ca^{2+} signal with the InsP_3 resulting from stimulation of the parallel fibres.

voltage-operated channels. In addition, there is a local release of NO. If the cGMP generated by NO stimulates the production of cADPR, as is the case in sea urchin eggs⁴, this dendritic calcium spike may be amplified by calcium release from the ryanodine-sensitive stores (Fig. 2). Second, if this climbing fibre-induced elevation of calcium within the dendrite spreads into the spine, it may serve to sensitize the resident InsP_3 receptors to the InsP_3 coming from the parallel-fibre input. Under normal conditions this parallel fibre-induced increase in InsP_3 fails to release calcium because it lacks its co-agonist (calcium), which is provided by the climbing fibres. The idea is that the InsP_3 receptors within the spine may function as the molecular coincidence detectors responsible for integrating the inputs from the climbing and parallel fibres (Fig. 2).

Finally, the interaction between cGMP and cADPR might explain the ability of NO and cGMP to amplify gene transcription in neuronal cells induced by calcium-dependent signalling pathways such as the opening of voltage-operated channels¹¹. This amplification was apparent only if the

two interacting pathways were activated within a narrow time window, further supporting the coincidence mechanism proposed in Fig. 2.

Although speculative, these examples emphasize the possible importance of coincidence detection and suggest ways in which cADPR and InsP_3 may generate complex calcium signals with very distinct spatial and temporal properties. □

Michael J. Berridge is in the AFRC Laboratory of Molecular Signalling, Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, UK.

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Plume origin for komatiites

Mike Bickle

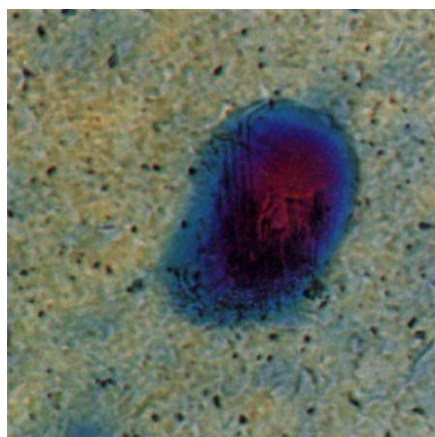
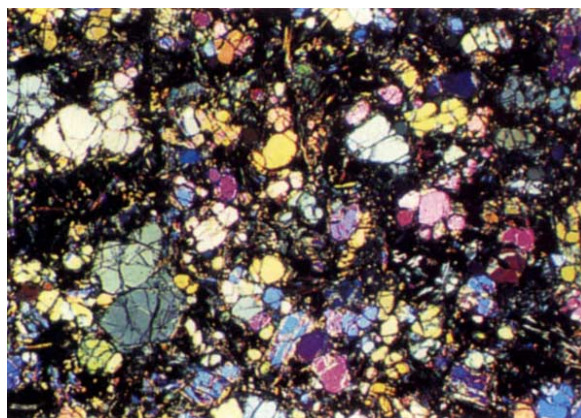
KOMATIITES, named for the Komati river in Transvaal, are the solid remains of the hottest lavas on Earth. As they are found only in rocks more than about 2.5 billion years old, they are direct evidence that the Earth's mantle was hotter during its early history. Most are poorly preserved, making it difficult to derive details of early temperatures and chemistry, so it was an unexpected boon when remarkably fresh komatiites were found in the Belingwe greenstone belt in Zimbabwe. Rare amongst komatiites, these contain the original unaltered olivine crystals rather than a *mélange* of secondary minerals, and better still, some of these crystals hold pristine melt preserved as glassy inclusions¹. McDonough and Ireland have now analysed the trace-element contents of the inclusions. Their results, reported on page 432 of this issue², suggest that komatiites originate in atypically hot upwelling plumes in the mantle.

Komatiites pose severe tests for the models of mantle melting that are proving successful in explaining present-day magmatism^{3,4}. Komatiites are rich in magnesium, yet this partitions preferentially into the solid rather than the liquid phase; to produce lavas with these magnesium contents, high degrees of melting and high temperatures are required. There have been suggestions that the original magnesium contents have been augmented since eruption, leading to serious overestimates of the eruption temperatures, but the textures, phenocryst compositions and geochemical variation point to an eruption temperature of about 1,580 °C for the most magnesian komatiites, with about 29 per cent MgO (see ref. 5 for a review). The hottest modern magmas reach only 1,400 °C or so.

An eruption temperature of 1,580 °C presents quite a problem. The source mantle from which the liquids rose would have been derived at depths of about 500 km, with a potential temperature of about 1,900 °C (ref. 5) (where temperature varies with pressure adiabatically, it is necessary to give the temperature at a reference pressure — here, 'potential temperature' would be the temperature at 1 atmosphere). If this represented average mantle conditions, much of the upper mantle would have been partially molten and the rapid advection of latent heat to the surface would have led to rapid cooling⁶. For this reason Nisbet and co-workers⁵ concurred with an earlier suggestion that komatiites are derived from upwelling plumes of material 200–300 °C hotter than the average Archaean mantle⁷. The plumes would be similar to, but hotter than, the plume that maintains

volcanism in the Hawaiian islands.

McDonough and Ireland's analyses, made with the high-resolution ion microprobe SHRIMP, add weight to this conclusion. Their samples are drawn from the glass inclusions trapped in the Belingwe komatiites, and include some of the most incompatible trace elements (barium, strontium and potassium) which are the



The Belingwe komatiites. Top, section showing the olivine phenocrysts, preserved virtually unaltered (field of view about 6.5 mm across). Bottom, photomicrograph of a 40 × 24-μm glass inclusion within olivine crystal from the lava, sample ZM15. The surrounding olivine has preserved the glass from hydration and alteration. The acicular crystals are clinopyroxene. The mauve to blue colour zonation of glass results from variable thickness of olivine below the inclusion, viewed between crossed polarizers.

most informative tectonically, but also the most easily altered. 'Incompatible' elements are those that partition almost entirely into the melt, and their concentrations show the most extreme variations between the various mantle reservoirs. These komatiites have higher Ba/K, Ba/La and K/La ratios than mid-ocean-ridge basalts, as plume-related magmas should, and they lack the higher K/Nb or Ba/Nb

ratios that would characterize crustal contamination or an island-arc origin (island arcs are belts of volcanic activity above a subduction zone). Of course, such a geochemical classification rests on the assumption that the mantle's trace-element enrichments in Archaean times were similar to those in modern mantle, but there is some evidence for this in other element ratios⁸.

The confirmation of a plume origin for the Belingwe komatiites is important. Komatiites are not representative of average-temperature mantle, they may be derived from mantle sources with atypical chemistry and they are not typical products of magmatism related to extension of the lithosphere, such as at mid-ocean ridges. If they do originate in a plume beneath a thick layer of overlying oceanic or continental lithosphere, this solves one other problem plaguing models of melting in the komatiite source region. Adiabatic upwelling of mantle at a potential temperature of 1,900 °C to

near the surface would produce melt thicknesses of 50 to 100 km (ref. 9), whereas real komatiite sequences are seldom more than a few hundred metres thick. Production of komatiites in plumes beneath thick lithosphere may have allowed the formation of smaller volumes of relatively hot magnesian magmas.

The more debatable conclusion of McDonough and Ireland is that the Belingwe lavas represent oceanic rather than continental plume magmatism. The Belingwe greenstone belt has an apparently simple stratigraphy with the volcanic succession overlying a thin sedimentary formation on older continental basement¹⁰. Recently, Kusky and Kidd¹¹ have reinterpreted the structural setting of the greenstone belt, suggesting instead that the volcanic unit is part of an ophiolite emplaced along a high-temperature shear zone. Ophiolites are fragmented sections of oceanic crust and mantle, often found thrust into place in modern mountain belts. Field studies have not described the kind of rocks expected to be associated with such a shear zone¹², but it is impossible to prove whether the original stratigraphy was continuous in sequence where exposure is incomplete and shear zones occur.

The geochemical evidence is equally uncertain. The new trace-element data show that crustal contamination of the more magnesian komatiite lavas is minimal; their K/Nb ratios limit it to less than half a per cent of their mass. This accords with previous interpretations of komatiite chemistry but does not rule out a con-

tinental setting. Chauvel *et al.*¹³ interpret the lead isotopic compositions of the associated komatiitic basalts (these are less-magnesian, lower-temperature lavas) to reflect contamination by about half a per cent of crust relative to the Belingwe komatiites. Also, xenocryst zircons derived from an older crustal basement are found in komatiitic basalts at Kambalda, western Australia¹⁴, yet the associated komatiites are chemically similar to the Belingwe lavas. The puzzle is how such high-temperature magmas can pass through thick continental crust with so little contamination¹⁵.

So where does this leave us? The trace-element data on the Belingwe komatiites are consistent with a plume-related origin

from atypically hot Archaean mantle, according well with predictions for the thermal structure of the Archaean mantle but showing that the greenstone-belt volcanics are not analogous to modern oceanic crust. Whether the plume impinging on oceanic or on continental lithosphere is more contentious and is unlikely to be solved by geochemistry alone. The struggle to unravel the stratigraphy and structural relationships will have to continue, unless discovery of zircon xenocrysts provides definitive evidence of an older continental basement. □

Mike Bickle is in the Department of Earth Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EQ, UK.

temperatures of the order of 100 millikelvins or less are required.

The island has to be connected, but very weakly, to the rest of the electrical circuit (Fig. 1), so as to allow electrons, paired or singly, to migrate. Lafarge *et al.* have used an aluminium island, a strip about 2 μm long and 100 nm wide, patterned from a 30-nm-thick film; a carefully grown high-resistance aluminium oxide layer allows electrons to leak through to a much larger normal metal electrode that acts as a reservoir. The island has a capacitance of about 10^{-15} F to the ground electrode; its charge, or equivalently the number of electrons n_{island} , can be measured by another superconducting device (not shown in Fig. 1) extremely weakly coupled to the island.

As the potential U is increased, equal and opposite charges Q and $-Q$ build up on the ground electrode and on the island-reservoir combination; both are large enough for thermal fluctuations to make Q increase continuously. However, Q is shared out between the island and the reservoir, and if the discreteness of charge were unimportant, as at high temperatures, n_{island} would vary continuously with U (Fig. 2). At low temperatures, when the electrostatic energy of the island becomes significant, n_{island} jumps discontinuously at well-defined values of its high-temperature thermal average value n_{av} . If the island is put into the normal state, by applying a small magnetic field, n_{island} jumps by one electron whenever n_{av} passes through a half-integer; in the superconducting state, n_{island} jumps by two as n_{av} passes through an odd-integer.

Not only do these experiments provide satisfying and direct confirmation of electron pairing in superconductors, but they also allow a detailed analysis of how n_{island} varies with n_{av} at intermediate magnetic fields, giving a fairly direct measurement of the superconducting energy gap Δ . Of course, Δ is well known for aluminium, and the value that Lafarge *et al.* obtain is in good agreement with that measured by

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QUANTUM ELECTRONICS

Superconducting Noah's Ark

David Caplin

THE universal characteristic of a superconductor that distinguishes it from a normal, non-superconducting metal is that the conduction electrons are paired. Soon after it was suggested in 1957 that pairing is the key ingredient for a successful explanation¹ of the long-standing puzzle of superconductivity, the existence of paired electrons was confirmed, albeit slightly indirectly^{2,3}: the magnetic flux threading a superconducting ring is quantized in units of h/q , where h is Planck's constant, and q is the electrical charge of the carriers; those experiments demonstrated that the unit is $h/2e$, not h/e . Recent developments in nanolithography⁴ have enabled metal dots to be patterned that are small enough for single

electrons to be counted entering or leaving. Now, Lafarge *et al.*^{5,6} have demonstrated that in a superconductor it is indeed electron pairs that migrate.

Everyday electrostatics shows that in an absolute sense, the electronic charge e is large. It's easy to transfer enough electrons, perhaps 10^6 or 10^8 , to a comb or a nylon sweater to build up sufficient electrostatic potential and associated electric field to generate a spark discharge. At the beginning of the century, Millikan⁷ was able to use the electrostatic force on an oil drop carrying a net charge of just one or two electrons to make the first absolute measurement of e .

The electrostatic potential energy E_{es} of an object carrying charge Q is equal to $Q^2/2C$, where C is the capacitance. The discreteness of the charge on a metallic object becomes important when E_{es} for a net charge of a single electron is made large compared with thermal energies $k_{\text{B}}T$ (where k_{B} is Boltzmann's constant), which would otherwise smear things out. For metal islands of submicrometre dimensions,

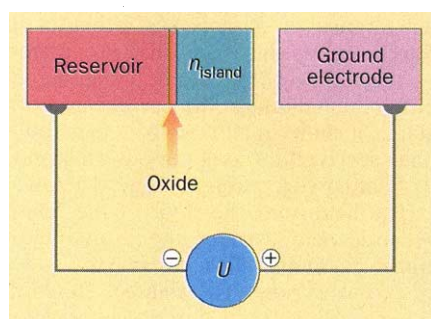


FIG. 1 Geometry of the submicrometre structure that allows electrons to be counted.

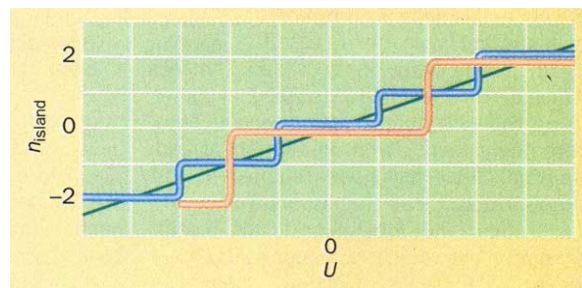


FIG. 2 At high temperatures, the number of electrons on the island, n_{island} , fluctuates rapidly, and its average value n_{av} changes continuously as the potential difference U is varied (green line). At low temperatures with the island superconductivity suppressed by a small magnetic field, n_{island} changes by one electron at a time (shown here schematically by the blue trace), but in the superconducting state the changes are pairwise (red trace).

other techniques. But the magnitude of the gap in heavy-fermion superconductors is still controversial, and in the high-temperature oxide materials, even more so. If similar structures could be fabricated from these exotic superconductors — and it's a big if — analogous experi-

ments could shed new light on the detailed nature of their superconductivity. □

David Caplin is at the Centre for High Temperature Superconductivity, Imperial College of Science, Technology and Medicine, London SW7 2BZ, UK.

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PALAEONTOLOGY

Roots for the guinea pig

Bob Savage

ON page 434 of this issue¹, Wyss and colleagues describe their analysis of a fossil fauna in the Chilean Andes which dates to around 34 million years ago. Among the remains are those of a rodent. The discovery takes back the arrival of rodents in South America by some 10 million years, and it provides support for the hypothesis that the group originated in Africa rather than North America.

South America was isolated for most of the Cenozoic, and for some 50 million years (Myr) the early mammals evolved and diversified there. Less than 5 Myr ago, an island chain and later an isthmus connected the continent to Central America. However, in deposits that are about 25 Myr old, there are representatives of two orders of mammals completely new to the continent, namely rodents and primates. These stocks are well known in older deposits in North America and the Old World. So the questions are when, whence and how did rodents reach South America while it was isolated.

The rise of the Andean mountain chain has been accompanied by abundant volcanism, resulting in, among other things, thick series of volcanoclastic deposits.

Using the rich mammal faunas collected from these beds, a succession of Land Mammal Ages has been established for the continent. But the succession is not continuous, the most significant hiatus occurring between the Eocene and Oligocene. The older, Eocene faunas (of Casamayoran and Mustersan Ages) are composed of marsupials, edentates and primitive members of South American herbivore families. The Oligocene faunas (Deseadan Age) are of new families evolved from the Eocene stocks, together with rodents and primates; the rodents are decidedly members of the caviomorphs,

the stock that includes guinea pigs and has since that time diversified through the continent. The boundaries of the Deseadan and Mustersan Ages are not well defined, however, and the time gap between them is believed to be between 15 and 25 Myr wide.

Wyss et al. now start to fill that gap. The

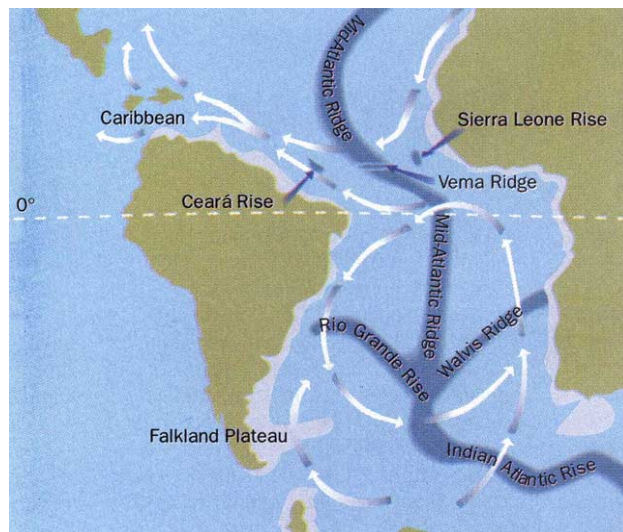
crown, as known in Early Oligocene rodents from Egypt, or from ancestors with a four-crested crown, as known in early North American rodents. The specimen discovered by Wyss et al. is known only from its mandibular dentition, but the wear pattern suggests it would have occluded with a five-crested upper molar. An African origin is also supported by karyological and molecular evidence from living rodents.

The source area of South American monkeys has posed similar problems. The discovery in the Bolivian Andes of *Branisella* in Deseadan beds dated at around 26 Myr old marks the earliest record^{2,3} (primates are absent at Tinguiririca, the site investigated by Wyss et al., which may be due to ecological reasons or may reflect a later arrival of the stock in South America). *Branisella* has the quadricuspid cheek teeth characteristic of present-day monkeys in the continent, whereas marmosets, another extant family of South American primates, have tricuspid teeth. That could mean that the marmosets' apparent primitiveness is secondarily derived or that the New World primates are not monophyletic.

For primates, the main African connection is with the Fayum stratum in Egypt, which has long been recorded as of Oligocene age. Recent estimates indicate that it is more than 31 Myr old, on the basis of isotopic dating of a capping lava³. The similarities of *Branisella* with some of the Fayum primates, particularly *Aegyptopithecus*, points to Africa for the origin of the New World monkeys⁴. A variant of this has both New and Old World monkeys originating in the Eocene of southeast Asia, where only a few poorly known specimens have as yet been described⁴. The Asian forms would have had to reach South America by way of Africa or North America, so it does not add another source area. A third possibility is that the South American monkeys are derived from an Eocene family in North America⁵.

Finally, the 'how'. Explanations for the appearance of rodents and primates in South America have one thing in common — they all invoke island-hopping, across either the Atlantic or the Caribbean. Both stocks include small mammals that could have survived for long periods on islands of floating vegetation in tropical zones.

The history of the floor of the South Atlantic demonstrates the evolution of transform faults which gave rise to a series of east–west ridges (see figure). The Mid-Atlantic Ridge itself rises above sea level in several island chains; St Paul's Rocks are almost on the Equator. Owing to the



The South Atlantic in the Early Oligocene (about 30 Myr ago), showing prevailing currents and oceanic rises, some of which may have been used during periods of lowered sea levels for island-hopping by rodents and primates to cross from Africa to South America. (Adapted from ref. 7.)

fauna they have recorded is tightly dated, being sandwiched between ashes that are 31–37 Myr old, which takes about 10 Myr off the top boundary of the hiatus and pushes back the arrival of rodents to the Late Eocene.

As regards the 'whence', the evidence outlined by Wyss and colleagues favours the idea that South American caviomorphs originated in Africa. Deseadan rodents (24–27 Myr old) of Bolivia clearly display the dental, skull and mandible characters of caviomorphs. Their upper molar tooth pattern could be derived either from ancestors with a five-crested

geography of the coasts of Brazil and west Africa, the equatorial zone of the Atlantic is the narrowest part today and overlap of the coasts in early Tertiary times would have greatly narrowed the sea crossing. The Ceará and Sierra Leone rises are equatorial, as is the Vema transform sliver, which is currently 600 metres below sea-level and is capped by Miocene reef limestones similar to those of the Bahamas⁶. Prevailing ocean currents would further have favoured drifting from Africa towards central South America. The history of the Caribbean region involves two volcanic chains between Central and South America that were active at various times during the Tertiary, and a major obstacle to southerly island-hopping would have been the strong east-west ocean currents between the Pacific and the Atlantic.

Because of the lack of isotopic dating for the Mustersan, there is still a gap of

unknown length (probably between 5 and 15 Myr) in our knowledge of mammal faunal sequences in South America. It was during this time that the rodents, and perhaps also the primates, reached the continent. □

Bob Savage is in the Department of Geology, University of Bristol, Wills Memorial Building, Queens Road, Bristol BS8 1RJ, UK.

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DOPAMINE RECEPTORS

The D4 and schizophrenia

Leslie L. Iversen

ONE of the achievements of research on mental illness in the past 20 years has been the recognition of the key role that brain dopamine systems play in mediating the actions of anti-schizophrenic drugs. The starting point was the observation that amphetamines, which can cause a schizophrenia-like psychosis in people, act by promoting the release of dopamine in the brain, and that anti-schizophrenic drugs (neuroleptics) potentially block the psychostimulant actions of amphetamines in animals and humans^{1,2}. The 'dopamine hypothesis' received further support when biochemical studies revealed directly that neuroleptic drugs act as antagonists at brain dopamine receptors of the D2 subtype^{3,4}.

The paper by Seeman and colleagues on page 441 of this issue⁵ may represent a further advance, one that is potentially of high significance. The conclusions to be drawn from this work are that the functional coupling of dopamine receptors may be abnormal in the brains of schizophrenic patients, and that this phenomenon is accompanied by a selective increase in the density of a particular D2-related receptor subtype, the dopamine D4 receptor.

Several previous studies, involving use of various radioligands in samples of post-mortem brain tissue, have examined the possibility that abnormally large numbers of dopamine D2 receptors might be present in the schizophrenic brain. Most such studies did indeed reveal increased density of D2 receptors. But the interpretation of the findings has remained

controversial, as before they died most patients had been treated with neuroleptic drugs, which are themselves known to cause increases in dopamine receptors in animal brain on chronic administration^{6–8}. Moreover, two studies in living patients, using positron emission tomography, gave conflicting results. Wong *et al.*⁹ found substantial increases in dopamine receptors in ten drug-naïve patients, using [¹¹C]spiperone as the radioligand. Farde *et al.*¹⁰, however, failed to find any such increases in 18 similar subjects, using [¹¹C]raclopride.

The results reported by Seeman *et al.* may help to reconcile some of these differences. The authors sought to measure the density of dopamine receptors of the D4 subtype, which is of particular interest because it is a possible target for the atypical anti-schizophrenic drug clozapine¹¹. In the absence of a radioligand with selectivity for D4 sites, they employed [³H]raclopride, which has high affinity for dopamine D2 and D3 receptors but not for D4, and [³H]emonaipride or [³H]spiperone, which bind D2, D3 and D4 sites with high affinity. The number of D4 sites is calculated as the difference between [³H]raclopride and [³H]emonaipride binding¹². According to this approach, D4 receptors represent a relatively minor proportion of the total dopamine receptor population in basal ganglia tissue from normal subjects, or from patients dying with Parkinson's or Alzheimer's disease. In samples from 32 patients who had been diagnosed as schizophrenic, however, there was a

sixfold increase in D4 receptor numbers, which in these cases represented up to 40 per cent of the total D2-like population.

In addition, Seeman and co-workers found that tissue from schizophrenic patients differed from that of control subjects, and from that of Huntington's or Parkinson's patients, in being unresponsive to guanine nucleotides. Guanine nucleotides normally cause an increase in [³H]raclopride binding, but the effect was not evident in the schizophrenia tissue samples. This observation suggests that there may be some alteration in the G-protein-mediated functional coupling of dopamine receptors in schizophrenia, which could be as important as the change in D4 receptor numbers.

The vexed question of whether these changes are inherent to the psychotic illness, or are merely the effects of chronic treatment with neuroleptic drugs, is addressed in two ways. The authors show data from 'drug-free' patients (six out of a total of 32 in the schizophrenia group), who exhibited similar changes. And they compare their results with data from patients dying with Alzheimer's or Huntington's disease, most of whom were treated in life with doses of neuroleptics similar to those used in the schizophrenia group, but failed to show any biochemical changes.

The history of biological research in schizophrenia is littered with the skeletons of once attractive hypotheses, and it will be essential to see whether the conclusions of Seeman and colleagues can be confirmed in other laboratories. Counting receptor numbers by estimating the difference between two large numbers is far from ideal, but the possible proliferation of dopamine D4 receptors in the brains of schizophrenic patients should be directly verifiable when selective dopamine D4 radioligands are developed. If the findings can be confirmed, the present paper will be a landmark on the way to a genuine improvement in our understanding of this enigmatic illness. □

Leslie L. Iversen is at Merck, Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Eastwick Road, Harlow, Essex CM20 2QR, UK.

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Those melting moments

D. J. Bishop

SHORTLY after the discovery of the high- T_c superconductors came the unwelcome surprise that even in modest magnetic fields these materials can have appreciable resistivities, low critical currents and reversible magnetizations which persist to quite low temperatures. This behaviour looked likely to jeopardize envisaged applications, so understanding its microscopic origins became one of the main goals of researchers in the field^{1,2}. On page 407 of this issue, Cubitt and colleagues³ show that small-angle neutron scattering experiments can provide a major step towards this understanding.

When a magnetic field is applied to a type II superconductor, one of three things can happen. The field can destroy the superconducting state (the normal state), it can be completely expelled from the superconductor (the Meissner state) or it can enter the sample in quantized bundles of magnetic flux called flux lines (the mixed state). In this mixed state, the flux lines can form a well-ordered lattice. Sketched in part *a* of the figure is the phase diagram for this behaviour in a conventional type II superconductor. The behaviour in this mixed state is important, as this is the high-field regime in which most of the hoped-for applications will operate.

The problem with high- T_c materials in this regime is acute. For example, in the compound known as Bi:2212 (named for its chemical formula, $\text{Bi}_{2.15}\text{Sr}_{1.95}\text{CaCu}_2\text{O}_{8+x}$), in fields of 1 tesla, the resistivity at temperatures above about 30 kelvin is no lower than that of a copper wire. This is surprising behaviour for a compound that has a low-field transition into a zero-resistance state at 90 kelvin and a very high upper critical field.

In 1988, Peter Gammel and I made a controversial suggestion^{4,5} to explain the anomalous high-temperature behaviour of the oxide superconductors. On the basis of high- Q mechanical oscillator data, we suggested that the high-temperature regime was a vortex liquid separated from the low-temperature vortex solid (lattice) by a melting transition, similar to the way ice melts into water. The high-temperature, high-resistance state would then arise because the rapid, thermally induced motion of the magnetic flux lines dissipates

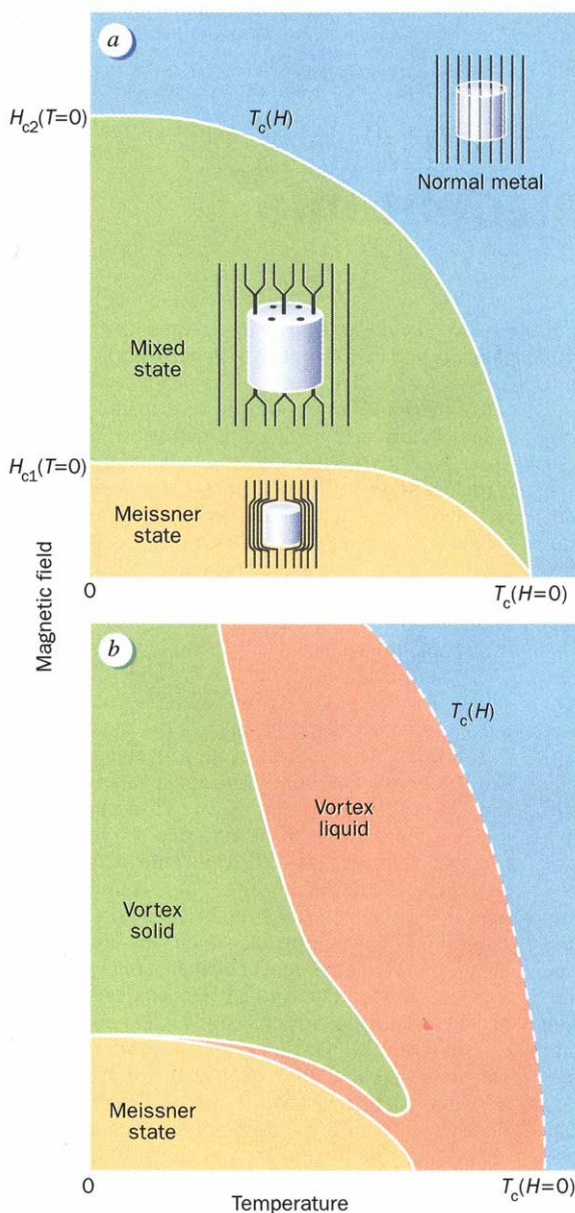
the energy in a flowing current. A modified phase diagram (part *b* of the figure) would be the result. This suggestion generated much controversy and a flood of papers for or against the idea. Cubitt and co-workers' experimental measurements, directly probing the magnetic flux lattice in one of these materials, provide some of the best evidence yet in its favour.

One of the problems in interpreting data on these materials is that most experimental probes provide only indirect information about the flux lattices them-

selves. Transport studies looking at carrier motion, which have provided compelling evidence for both a vortex-glass second-order transition⁶⁻⁸ in dirty samples and a first-order melting transition⁹ in clean samples, do not provide detailed structural information. Magnetic decoration can image the lattices at low temperatures, but provides only limited information at the higher temperatures at which they melt. Most other techniques either do not image the lattice at all or do so in a way that is very limited as a function of temperature and field.

Small-angle neutron scattering does not suffer from these limitations. The technique, first suggested by de Gennes and Matricon¹⁰, and put into practice by Cribier *et al.*¹¹, works by scattering neutrons from the small field modulations in the sample which are there because of the quantized flux lines of the mixed state. In much the same way that one can image lattices of atoms in crystals and obtain their structures, one can image the lattices of magnetic flux lines in type II superconductors and obtain their structures as a function of temperature and applied magnetic field. The drawback is that the signals can be very weak, especially for materials such as these which have long magnetic penetration lengths. Large, high-quality single crystals are then essential, and the new measurements represent a triumph in the art of sample growing as well as in measurement technique.

Cubitt and collaborators use small-angle neutron scattering to find direct evidence for melting of the magnetic flux lattice in Bi:2212. At low temperatures and fields, they see a good flux lattice characterized by six sharp peaks in reciprocal space of the scattered intensity. As they raise the temperature, they see these peaks abruptly disappear as the lattice melts. In addition, the peaks disappear as the magnetic field is increased. This last result looks as if it is a transition from three-dimensional flux lines (long connected lines) at low fields to two-dimensional 'pancake' vortices¹² at high fields. At low fields, when the vortices are far apart, their interactions within the copper oxide planes are weak in comparison to their interactions between planes and they are then three-dimensional. At high fields, however, the in-plane interactions should dominate, and the vortices should then become two-dimensional. For Bi:2212, it seems that this transition takes place at a



Phase diagram for conventional type II superconductors (*a*); and as modified for the oxide superconductors (*b*). The increased importance of thermal fluctuations in these systems accounts for the large vortex liquid regime in the oxide superconductors.

field strength of about 0.1 tesla.

The issue of flux-lattice melting is an important one for a number of reasons. Transport measurements show that when the flux lattice melts, the critical currents drop to zero and the resistivity becomes large, creating problems for many applications. Flux-lattice melting may also tell us much about basic issues such as phase transitions in the presence of disorder,

dimensional crossovers and line liquids. Experiments such as that of Cubitt and colleagues will go a long way in helping us to understand flux-lattice melting at a microscopic level. The researchers are to be congratulated on their achievement. □

D. J. Bishop is at AT&T Bell Laboratories, Murray Hill, New Jersey 07974, USA.

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HIV

Cyclophilins unfold the Gag?

P. J. Klasse, Thomas F. Schulz and Keith R. Willison

WRITING in *Cell*¹, Luban *et al.* report that the Gag protein of human immunodeficiency virus type 1 (HIV-1) binds to cyclophilins A and B. This work brings together aspects of immunology, protein folding and retrovirology, and raises a host of significant questions.

First, cyclophilins are the intracellular receptors for the immunosuppressive drug cyclosporin A (CysA)². Second, they are peptidyl-prolyl-*cis-trans* isomerases (PPIases), which assist in the intracellular folding of proteins². Third, the virally encoded Gag proteins have been postulated to interact with unknown host-cell factors, both early and late in the retroviral life-cycle³; so we now have the possibility that cyclophilins are such factors. Furthermore, a type of murine immunodeficiency induced by the Duplan strain of radiation murine leukaemia virus (MuLV) has been attributed to a defective form of the virus with an aberrant Gag protein⁴. Could the Gag protein of HIV-1 also be immunosuppressive?

Luban *et al.* discovered the Gag–cyclophilin interaction by screening a human complementary DNA library in a two-hybrid interactive cloning system. They also demonstrate that CysA competes with Gag for binding to the cyclophilins. Might, then, the Gag protein have similar effects to those of CysA? Immunosuppression induced by CysA is thought to be mediated by the binding of the CysA–cyclophilin complex to calcineurin, a phosphatase involved in T-cell activation². Luban *et al.* show that the Gag–cyclophilin complex does not bind to calcineurin, and suggest that the HIV-1 protein may mediate immunosuppression by other mechanisms (for example by inhibiting the binding of the cyclophilins to a previously described, possibly

physiological ligand).

By using deletion mutants, they delimited the part of the Gag precursor involved in cyclophilin binding to a region in the amino-terminal two-thirds of the molecule (Fig. 1). However, free capsid protein, p24, also binds to the cyclophilins. Luban *et al.* point out that cyclophilin binding might therefore have

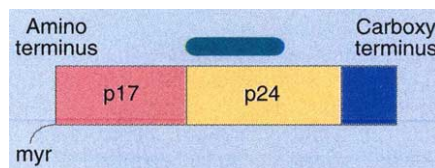


FIG. 1 Map of the HIV-1 Gag precursor showing the regions corresponding to the matrix protein (p17) and capsid protein (p24), and the approximate region necessary for cyclophilin binding (green). Other smaller Gag proteins are represented by the blue box. myr, myristyl group.

some function early in the viral life cycle, when the proteolytically processed Gag proteins enter the cytoplasm, or late in the cycle, when the precursor assembles and directs the budding of new virus particles.

Here, the possible involvement of cyclophilins in HIV replication is considered against the background of their PPIase activity, their three-dimensional structure and the hypothetical role of other host factors in retroviral Gag functions. First we deal with possible interactions between Gag and host proteins at the stages before DNA integration.

Early in the retroviral life-cycle the fusion of the viral envelope with the host-cell membrane delivers a Gag particle, which contains the RNA genome and enzymes, into the cytoplasm of a susceptible cell³. Host-cell factors may be in-

involved in uncoating of the Gag particle, and in the transport of viral proteins complexed with newly synthesized proviral DNA copies to the cell nucleus, where the DNA is integrated into the host-cell genome⁵. What function could cyclophilin binding have early in the replicative cycle of HIV-1? As both the Gag precursor and the capsid protein p24 can bind cyclophilin, it does not seem to be in differentiating the incoming Gag–protein movement in the cytoplasm from that of the newly synthesized precursor. If cyclophilin is involved in uncoating, however, the PPIase activity may provide a mechanism by catalysing the unfolding reaction.

How general are the Gag–cyclophilin interactions? Luban *et al.* find that the Gag precursor of the simian immunodeficiency virus (SIV) binds to cyclophilin B, but not A, and that CysA competes more efficiently with HIV-1 Gag for binding to cyclophilin A than B. However, cyclophilin B resides within the cisternae of the endoplasmic reticulum, but cyclophilin A, like the incoming and the newly synthesized Gag proteins, is cytoplasmic^{2,3}. It will thus be interesting to see how the capsid protein of HIV-2 interacts with cyclophilins.

The Gag proteins of Moloney-MuLV (a type-C virus) and Mazon–Pfizer monkey virus (type-D) bind neither to cyclophilin A nor B¹. Thus, although the cyclophilins are highly conserved evolutionarily, they do not interact generally with retroviral Gag proteins. Earlier studies of MuLV have, though, pointed to host-factor interactions with Gag proteins. Different capsid proteins determine the tropism of MuLV for cells carrying either the *Fv-Iⁿ* or *Fv-I^b* allele⁵. A host protein that binds to MuLV Gag has been identified, but it binds to Gag from MuLV strains with either tropism. Its gene and the *Fv-I* locus are on different mouse chromosomes⁶. Although this Gag-interacting protein is not directly responsible for the *Fv-I* restriction, it may nevertheless be involved in MuLV replication, corresponding to a possible role for cyclophilin in HIV replication.

What of the folding, assembly and budding stages? Gag precursors are synthesized and folded in the cytoplasm of infected cells. Type-B and -D retroviruses form cytoplasmic core particles that migrate to the cell membrane. Other retroviruses, including HIV, assemble their cores at the cell membrane³. In both cases there is the possibility of interactions with cellular factors for transport and attachment to, and inclusion in, the cell membrane. Contacts between the Gag proteins and the viral envelope glycoproteins may occur, but they are not required for budding to take place. Myristylation of the matrix protein (Fig. 1) is necessary for the budding of some retroviruses. Tagging

ten residues from pp60^{src} onto a truncated, nonfunctional Gag precursor of Rous sarcoma virus can restore budding ability by directing the hybrid protein to the membrane-associated pp60^{src} receptor³. It has therefore been hypothesized that there are receptors for the Gag precursors on the inside of the cell membrane³. However, mature cyclophilin A and B are not membrane-anchored so, in accordance with their

capsid-protein model only at one. The part of Gag required for cyclophilin binding corresponds to the B-F strands in the SIV-HIV model, including the α -helix and a protruding region⁹. Notably, the E strand and the protruding region contain three and five prolyl residues out of 25 in SIV and HIV-1 Gag, respectively.

A test of the importance of cyclophilin in Gag-directed budding may be near at hand. A deletion mutant of HIV-1, lack-

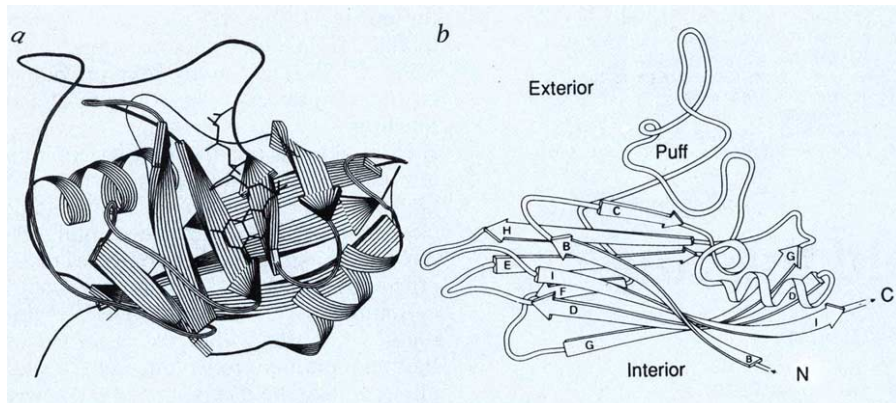


FIG. 2 *a*, Ribbon diagram of the eight-stranded β -barrel structure of cyclophilin (from ref. 7). The two α -helices are shown as spirals and the β -strands as arrows pointing in the amino-to-carboxy-terminal direction. A proline-containing peptide is situated in the catalytic cleft (left). *b*, Model of the SIV-HIV capsid protein, which has a similar overall structure (from ref. 9). The strands of the β -barrel are labelled consecutively from B to I. One α -helix connects strands C and D (right), as in cyclophilin. A protruding region between strands E and F (Puff) corresponds to part of the cyclophilin-binding region in HIV-1 p24. A difference between the two structures is that the E strand is antiparallel to the F strand, as is the G to the H, in the SIV-HIV model; the corresponding strands in the cyclophilin structure are parallel.

PPIase activity, a more plausible function for them may be the catalysis of Gag folding. The presence of cyclophilin A in the cytoplasm is consistent with this.

CysA, a cyclical undecapeptide, binds to the catalytic site of the cyclophilins^{2,7}; furthermore, competition between CysA and HIV-1 Gag in binding to the cyclophilins suggests that the two molecules have overlapping binding sites¹. Alternatively, CysA could inhibit Gag binding allosterically, although the conformational changes induced by CysA are small⁷. Even if part of Gag binds to the catalytic cleft, it remains to be seen whether peptidyl-prolyl-*cis-trans* isomerization of Gag is thereby catalysed. There are other plausible functions for the interaction: the Gag proteins are hydrophobic, and cyclophilin binding may keep them from inadvertent contacts with cellular proteins.

The three-dimensional structure of cyclophilin, with and without bound CysA, has been solved by X-ray crystallography and nuclear magnetic resonance^{7,8} (Fig. 2). The structure of the capsid protein of SIV and HIV has been modelled on the picornaviral VP2 coat protein⁹. Both the cyclophilin and the capsid-protein structure consist of eight-stranded β -barrels, cyclophilin having α -helices at both apertures of the barrel, the

ing 56 amino-acid residues in the region of Gag to which the cyclophilin binding has been mapped, is fully competent in Gag assembly and virus-particle formation, but the virions are not infectious¹⁰. If this Gag mutant does not bind to the cyclophilins, then other functions of the interaction will have to be sought, earlier in the replication cycle.

It remains an open question whether the cyclophilin-Gag binding is involved in viral replication or immunosuppression; such binding may even be to the disadvantage of viral growth. In either case, it will inspire therapeutic strategies to nip HIV in the bud. □

P. J. Klasse, Thomas F. Schulz and Keith R. Willison are in the Chester Beatty Laboratories, Institute of Cancer Research, Fulham Road, London SW3 6JB, UK.

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Carbon polymers

MANY carbon compounds burn or decompose to soot. This apparently simply reaction is in fact highly subtle. Its earlier stages may pass through closed-shell graphite molecules of the fullerene type, a hot research topic these days. Accordingly, Daedalus is looking anew at soot-generating reactions.

He points out that in the gas phase, acetylene reacts vigorously with chlorine, producing hydrogen chloride and soot. When dissolved in an inert solvent, however, these substances react in a more subdued manner to give chlorinated ethane and ethylene derivatives. So intrepid DREADCO chemists are reacting acetylene and chlorine in inert media like liquid xenon. The critical point of xenon is 16 °C at 58 atmospheres, so the reaction can be taken smoothly from liquid phase through criticality to gas phase simply by varying the pressure. Daedalus wants to map out the transition between the chlorination and soot-forming reactions, and to explore the outer fringes of the latter. Where it cannot go to completion, it should choke off to leave all sorts of intermediate stages: fullerenes, micro-diamonds, carbon nanotubes and so on. They may be pure carbon polymers, or novel carbon-rich fragments whose dangling bonds have been chlorinated or hydrogenated.

The reaction should be particularly interesting in the critical region. The density of a critical fluid varies wildly from point to point, on a scale that is highly sensitive to temperature. A small temperature change can swing its scale of granularity from a micrometre or so right down to a few atomic diameters. Daedalus reckons that a reaction going in such a 'critically opalescent' medium should tend to produce molecules that fit neatly into the fluctuations. If so, careful control of the temperature of the reaction might selectively generate carbon polymers of well-defined molecular size. This could be a route to the giant fullerenes, those huge hollow closed-shell graphite structures which have so far evaded synthesis.

The DREADCO team will be especially alert for crazy new carbon structures, like polyfullerenes, screw-dislocated graphite (the ultimate extension of the helicene series of molecules), and branched, knotted or toroidal carbon nanotubes. Daedalus also recalls Ostwald's rule, that an allotrope condenses from the vapour into the less-stable solid form. As the reaction will occur in the graphitic part of the carbon phase-diagram, there is a good chance that bulk diamond will crystallize from it.

David Jones

Ultraviolet vision in lizards

SIR — There is evidence that each of the main vertebrate groups can detect ultraviolet light¹, but, in contrast to the situation in invertebrates, the role and/or importance of ultraviolet vision in vertebrates is under debate¹. One suggestion is that ultraviolet vision may be important in communication, but evidence to support this function is limited to the fact that the bodies of some fish and birds are highly reflective in ultraviolet light^{2,3}. We have been studying five closely related species of anoline lizards from Puerto Rico, and find evidence that ultraviolet vision is important in their system of visual communication, which is based on displays consisting of motion patterns of the body and a colourful, ex-

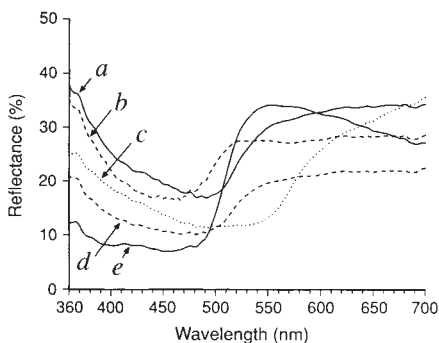


FIG. 1 Reflectance spectra (per cent reflectance compared to that from a magnesium carbonate white standard) from the dewlaps of *a*, *Anolis krugi*; *b*, *A. cristatellus*; *c*, *A. pulchellus*; *d*, *A. gundlachi* and *e*, *A. evermanni*.

pandable throat fan called the dewlap.

As part of a comprehensive study of the anoline retina (manuscript in preparation), we carried out microspectrophotometry and discovered that each of these five species has four classes of cones, with absorption maxima at about 565, 495, 450 and 365 nm. The ultraviolet-sensitive cone ($\lambda_{\max}=365$ nm) is always associated with a colourless oil droplet, which is transparent (optical density <0.2) down to the shortest wavelengths measured (320 nm), as were the lens and cornea. This is the first reported ultraviolet photoreceptor in any reptile, although earlier behavioural

studies have suggested that there is ultraviolet visual capacity in one turtle⁴ and one lizard⁵ species.

We measured the spectral reflectance of the dewlaps (Fig. 1), and found that two species exhibited a high degree of ultraviolet reflectance (>35 per cent), two exhibited low reflectance (<25 per cent), and the third was intermediate. We photographed each dewlap in natural sunlight with an ultraviolet-sensitive video camera under two conditions: with a filter transmitting only in the near-ultraviolet and with a filter transmitting in the range of normal human vision (Fig. 2). As expected from Fig. 1, the dewlaps of *Anolis cristatellus* and *A. krugi* appeared very bright in ultraviolet light, whereas those of *A. evermanni* and *A. gundlachi* appeared dark. The dewlap of *A. pulchellus* has a distinct pattern in the ultraviolet — half dark, half bright — which explains why it appears to have an intermediate level of reflectance in Fig. 1. The photographs also revealed a bright spot at the corner of the mouth visible in all five species only when the mouth is open — a typical anoline threat display (Fig. 2).

There seems to be a relationship between the light conditions typical of the microhabitat of each species and the extent of dewlap ultraviolet reflectance. The three species with ultraviolet-bright dewlaps come from microhabitats which are often exposed to direct sunlight and/or blue sky⁶, in which there is a great deal of ultraviolet light⁷. The two species with ultraviolet-dark dewlaps inhabit the understorey of closed-canopy forest^{6,8}, which contains little ultraviolet light⁷. Thus, in habitats where there is sufficient ultraviolet, an ultraviolet-reflective dewlap will appear bright and contrast sharply with the background vegetation, which reflects very little ultraviolet light. We believe that ultraviolet dewlap reflectance did not evolve (or was lost) in the two forest species because it would do little to enhance the visibility of the dewlap. The presence of ultraviolet visual receptors in the forest species may be due to phylogenetic inertia, or they may be for some other purpose (for example, prey detection near light gaps).

Our results strongly suggest that ultraviolet vision is involved in anoline communication because: (1) dewlaps are used almost exclusively in communication and hidden at other times; (2) only species

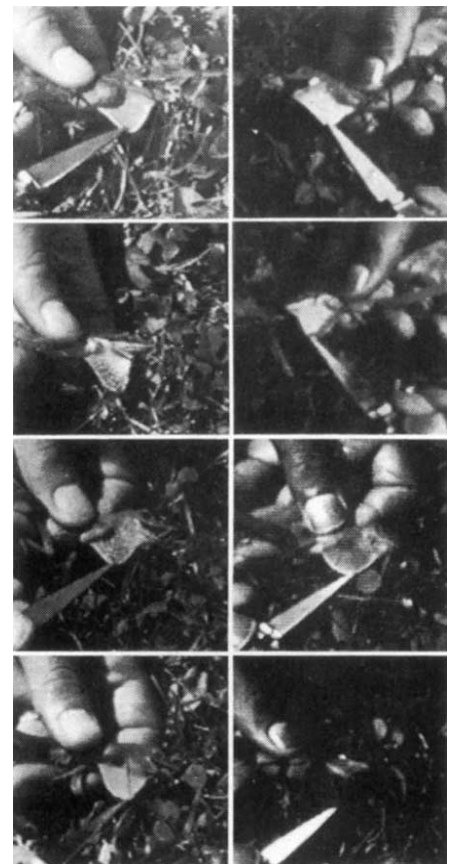


FIG. 2 An ultraviolet-enhanced video camera (CIDTEC) with a Flectan quartz reflecting lens (Nye Optical) was used to record the appearance of dewlaps under natural daylight conditions against a background of green grass. The first photograph in each row was made with filters passing light in the spectral range 400–680 nm. The second photograph in each row shows the relative brightness in the near-ultraviolet (320–400 nm) as isolated using a Hoya U360 filter plus an infrared blocker (Omega Optical). Contrast for the two spectral regions was equalized against a set of calibrated reflectance standards. The species shown are (top to bottom) *A. cristatellus*, *A. pulchellus*, *A. gundlachi* and *A. evermanni*. *A. krugi* (not shown) is similar in appearance to *A. cristatellus*.

from ultraviolet-rich habitats possess ultraviolet-reflective dewlaps; and (3) only the dewlaps, and not the rest of the body, are ultraviolet-reflective.

Leo J. Fleishman

Department of Biological Sciences,
Union College,
Schenectady,
New York 12308, USA

Ellis R. Loew

Department of Physiology,
College of Veterinary Medicine,
Cornell University,
Ithaca,
New York 14853, USA

Manuel Leal

Department of Biology,
University of Puerto Rico,
Rio Piedras,
Puerto Rico 00931-3360, USA

Scientific Correspondence

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C₇₀ in solvent mixtures

SIR — The fullerene (5,6)-C₇₀ exhibits unusual solvatochromism in acetonitrile–toluene mixtures at room temperature. At a C₇₀ concentration of 6.6×10^{-6} M, an increase of acetonitrile volume fraction (x_{ace}) in the mixtures up to 60% causes essentially no spectroscopic change of the

out the structures in the 300–400-nm region. In addition, the spectral shape change is reversible, and accompanied by a significant increase in molar absorptivity, corresponding to much enhanced transition probabilities. The purple solution is macroscopically homogeneous and transparent, and is thermodynamically stable, with no changes in time or through sonication.

The observed colour and spectral changes are obviously not a typical result of solvatochromism. The phenomenon can be attributed to the formation of a new absorbing species at $60\% \leq x_{\text{ace}} \leq 70\%$. A further increase of x_{ace} from 70 to 97% does not fundamentally change the absorption spectrum any further (part A in the figure), but apparently enhances the absorption of the new species. From a C₇₀ concentration dependence study, we find that the formation of the new species depends strongly on the solution concentration (part B in the figure). The lower the concentration, the smaller is the contribution of the new species. For a very dilute C₇₀ solution ($\leq 8 \times 10^{-8}$ M), the absorption spectrum at $x_{\text{ace}} = 70\%$ is close to the spectrum in neat toluene. The results indicate that the formation of the new species involves more than one C₇₀ molecule.

We are reluctant to assign the new species to C₇₀ dimers. Rather, it is more likely that the purple solution is a result of absorption by a distribution of solvated C₇₀ clusters. We use the term 'cluster' (which refers to an aggregate of a few C₇₀ molecules) because the corresponding solution is quite different from a conventional colloidal solution. The purple solution formed at $x_{\text{ace}} = 70\%$ is macroscopically indistinguishable

from a dilute solution of C₇₀ in neat toluene, except for the difference in colour (the C₇₀ toluene solution is reddish orange).

The formation of C₇₀ clusters in a solvent mixture can probably be represented by a microscopic picture similar to the one for micelles. Because of rather poor solubility of C₇₀ in acetonitrile, the C₇₀ clusters are probably surrounded by shells of toluene molecules, which maintain the stability of the clusters in a

homogeneous solution. Although the formation of clusters in such a low concentration room-temperature solution is clearly a result of vastly different solubilities of C₇₀ in acetonitrile and toluene, it is a rather unusual phenomenon that has not been observed in other systems, and is probably dictated by the shape, dimension and other intrinsic properties of C₇₀ molecules^{1,2}. Absorption of C₇₀ clusters is also observed in other solvent mixtures composed of two components in which C₇₀ has very different solubilities, such as room-temperature mixtures of hexane and liquid carbon dioxide at about 150 bar.

The properties of the C₇₀ clusters should be close to those of solid-state C₇₀. Our observation thus offers an attractive alternative for the investigation and application of C₇₀ solid-state properties^{3–5}. In addition, the characteristic and reversible solvatochromism of C₇₀ may be valuable in optical-switching applications.

Ya-Ping Sun

Christopher E. Bunker

Department of Chemistry,
Clemson University,
Clemson,
South Carolina 29634, USA

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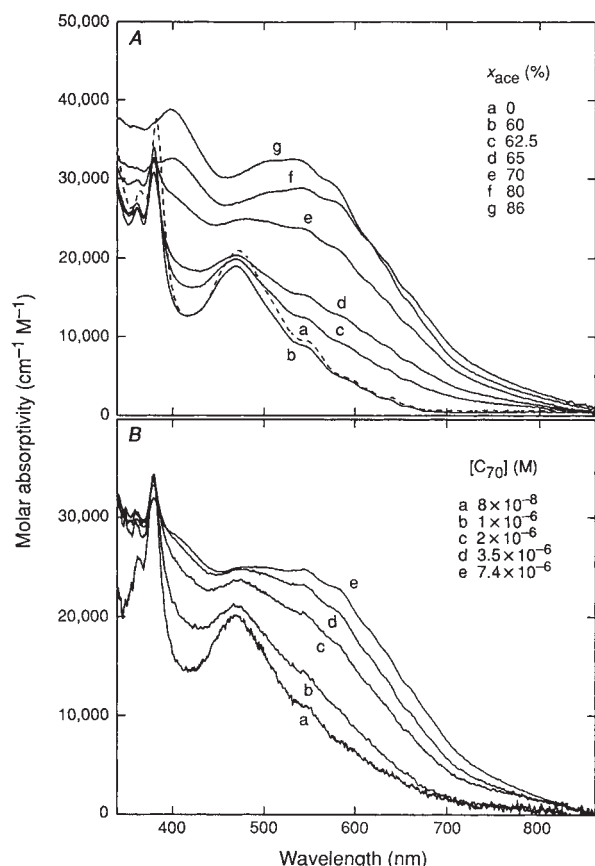
Split second?

SIR — In modern Olympic athletic events it is now customary to time competitors to an accuracy of 0.01 second — a few hundredths of a second can be crucial in deciding which athlete comes first. It seems to have gone unnoticed, however, that the usual starting procedure, which depends on a starting pistol, introduces differential delays owing to the finite speed of sound.

The starter usually stands just behind the line of athletes, close to the innermost lane (lane one). The loudspeakers placed in the blocks are primarily used to tell the athletes to go to their starting positions. The athletes run on hearing the shot. The typical width of a lane is 1 metre and the velocity of sound 340 metres per second, so an athlete in lane eight will hear the shot 0.02 seconds later than an athlete in lane one. Can we, then, be sure that we have correctly identified the winner in the event of a close finish?

Ramanand Jha

Department of Physics,
Indian Institute of Science,
Bangalore-560, 012 India



Absorption spectra of C₇₀: A, in room-temperature acetonitrile–toluene mixtures as a function of acetonitrile volume fraction (x_{ace}); and B, in acetonitrile (70%)–toluene (30%) mixture as a function of C₇₀ concentration. C₇₀ is from Aldrich with an estimated purity >98%. Acetonitrile and toluene, both from B&J (spectrophotometry grade), are used as received because no interference from possible impurities in the wavelength region of interest is found on the basis of absorption and emission spectroscopic measurements of the solvents. Absorption spectra are obtained on a computer-controlled Shimadzu UV2101-PC UV-Vis spectrophotometer.

solution. The absorption spectrum at $x_{\text{ace}} = 60\%$ is almost the same as the spectrum in neat toluene, except for a very minor blueshift (part A in the figure). But, as x_{ace} increases from 60 to 70%, the C₇₀ solution undergoes a dramatic colour change, from reddish orange to pinkish purple, and the corresponding absorption spectra show completely different features. The spectrum of the purple solution ($x_{\text{ace}} = 70\%$) is much broader, with substantial absorption in the 550–800-nm region and with-

Comings and goings

Frank Gonzalez-Crussi

The Beast Within: A History of the Werewolf. By Adam Douglas. *Chapmans/Orion*: 1992. Pp. 294. £15.95 (hbk); £5.99 (pbk).

Monstrous Imagination. By Marie-Hélène Huet. *Harvard University Press*: 1993. Pp. 316. \$59.95, £39.95 (hbk); \$23.95, £15.95 (pbk).

LYCANTHROPY (from the Greek *lykos*, wolf, and *anthropos*, man), the transformation of man into a wolf, strikes modern sensibilities as sheer nonsense or effete superstition. Yet impatience seems misplaced for ideas of millenarian perdurability and continuing influence. In *The Beast Within*, Adam Douglas informs us that since classical antiquity the nosology and symptomatology of lycanthropy have been firmly established. In Petronius's *Trimalchio's Banquet*, all the elements are found that were later made widely known through popular cinematography. The sufferer, whose characteristic habitus includes eyebrows that meet in the middle, experiences premonitory symptoms, triggered by light from a full moon, such as anxiety and an invincible tendency to seek secluded places in woods or cemeteries. Diagnostic signs then manifest themselves: the sprouting of a bristly fur, the growth of jutting fangs, a howling cry and behavioural modifications of rather unfriendly nuance. The disease runs a chronic course: attacks are self-limited and complete remissions occur, but relapses are the rule and there is no known therapy.

Written in the style of a popular book, this work nonetheless displays an admirable breadth. It is by no means a disappointment to scholars. Historians may find in the specialized literature a richer treatment of, say, the late sixteenth-century werewolf trials of the Franche-Comté, but rarely will they encounter a single source that collates research on the origin of the myth, its recurrence in disparate areas of the world, its appropriation by the movie industry, the remarkable occurrence of sporadic and epidemic insanity of a lycanthropic cast in our own century, and the gallant efforts to reconcile an apparent absurdity with contemporary medical notions. Cap-à-pie in straightforward, unassuming prose, Douglas rescues an ancient myth from the monstrous maw of pedantry, and manages to perform the dual feat that so many try in vain: to delight and to inform.

Monstrous Imagination is clearly more ambitious. Marie-Hélène Huet sets out to canvass not a specific instance of portentous transformation, but the much larger quandaries posed by the advent into the world of beings whose presence seems to challenge the unvarying order of nature. Her starting point is a survey of the

ancient belief in the power of maternal imagination to leave its imprint on the developing fetus. To the paralogical mind, woman is the potter, the fetus is the clay: a pregnant woman sits under a cherry tree, is startled by the sudden fall of cherries and delivers an infant covered by cherry-like hemangiomas; another, after gazing obsessively at a picture of St John the Baptist clad in a hermit's fur, brings forth into the world an excessively hirsute

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Werewolf painted by S. H. Vedder, 1901.

child. Under a sustained passion or a violent agitation, the maternal imagination erases the pristine genetic plan that decrees that the offspring must resemble its parents (thereby fulfilling Aristotle's central criterion in his definition of monstrosity), and imposes novel features on the progeny.

But the ability to mould the progeny, like the potter her clay, inevitably evokes a parallel between procreation and art. Therefore, in the second part of her work, Huet carries her investigation into the realm of literary aesthetics. In a show of dazzling erudition, she develops the thesis that Romantic aesthetic theory viewed literary creation as generation; and just as the maternal imagination had

been formerly believed capable of erasing all traces of the father's contribution to conception, so now Romanticism "reassigned the *vis imaginativa* to the father alone". In any case, the dark desire to reproduce without the other, Huet contends, underlies old biomedical interpretations of teratology as well as varied artistic works ranging from Madame Tussaud's wax sculptures to Mary Shelley's novel *Frankenstein* or the ancient legend of the golem.

The title of a haunting, powerful engraving of Goya, "The dream of the imagination produces monsters", describes equally well the theme of these two books. To face a confusing, disconsolate world, the reasoning faculty coins abstractions and generalizations. But man's yearnings, hopes and dreams have always manifested themselves in images, and these are sometimes terrifying. Nor is the symbolic 'imaging consciousness' an exclusive attribute of artists, children and psychopaths: it is a constitutive part of the human condition. Its function is to open and to maintain the communication between present-day man and his myths, perennial sources of life. This may seem too high-sounding when we think of the appalling trivialization of a legend in such films as *I Was a Teenage Werewolf*. But however banal and degraded it has been made by today's media, the werewolf remains a true myth because it was not created as a symbolic representation; it was not designed as a generalization derived from the varied materials of empirical experience, but as a description of what passed for a 'true occurrence' outside the sphere of everyday life. It has been said that all myths embody a nostalgia for a mythified, ahistoric or prehistoric past. So it is with lycanthropy, which flashes back to the time when man looked at the wolf with reverential awe and worshipped in

it the superior hunter and scavenger that he could not be. Thus, every time the story of the beast within is retold, expression is lent to the sadness ensconced in every human existence for what could have been and was not, the melancholy in not being able to become something else save by ceasing to be what we are.

Monstrous Imagination may also be said to advert to a perennial myth. Hypostatized in the idea of single-parent reproduction is a yearning for the mythical time when our kind was not cleft in two sexes: the desire for identification with Unity, for a return to the bliss and perfection of eternal Oneness. But the purport of this investigation is wider. It makes it apparent that even though 'monster' and 'monstros-

Mary Evans

ity' are concepts banned by science, they remain ineradicable from the imaging consciousness, for which every anomalous or deviant birth reasserts the equivocal nature of the human condition. Our imagination cannot help asking: angel or beast? And the reply is, neither one nor the other; but not a third one either, not a *tertium quid* between these two. Is it both at the same time, or alternatively first one and then the other? Perchance a hybrid, a chimera, like the Ravenna monster that Huet describes so well: angel by the wings, fish by the scales, and goat by the hoofs.

All those interested in the history of

ideas, myths and symbols should welcome the appearance of these two books. Both show that only the imagination truly perceives the elusiveness of our nature. For the human element will not be captured whole in the normal state or in the extremes of deviancy. At any level at which one approaches it, human nature seems to be passing through: "not always going," wrote Pascal, "but with comings and goings" (*Pensées II*, 27–354). □

Frank Gonzalez-Crussi is in the Department of Pathology, Children's Memorial Hospital, Chicago, Illinois 60614, USA.

Biological thought in development

Jack Cohen

Life Cycles: Reflections of an Evolutionary Biologist. By John Tyler Bonner. Princeton University Press: 1993. Pp. 209. \$19.95, £14.

NEARLY all working scientists spend their time and effort asking the next 'how?' question. On the rare occasions when they can step back from their work — from the new grant application, research report, student admission form, time sheet and so on — they can attempt to see their work in context, to ask the big 'why?' questions. Most of us don't get a chance to do this until we retire, and then much of the motive has been lost. A few biologists have managed to maintain the large view throughout their working lives, and have even written about their perspectives in books and reviews for the rest of us.

This feat seems to require an unusual combination of luck and skill from the outset. The luck comes from finding an organism with a peculiar habit, morphology or physiology: the giant nerve fibre in the cuttlefish, the peculiar mating habits of the stickleback, the geometry of the posterior clamp of monogenean fish parasites — all have launched new schools of investigation and illuminated biology more widely than might have been thought. Most biologists, however, begin with animals that sit square in the middle of our classificatory systems: ordinary mammals such as the rat; fish such as trout; and protozoa such as paramecium, easy to keep, and cheap too. Any special trick that these creatures use has unfortunately already been worked out.

John Tyler Bonner, by contrast, started with cellular slime moulds, whose disgusting name hides beautifully perverse organisms that totter on the edge of several biological assumptions. They are not unicellular, multicellular or cellular: individual amoebae live in the soil (or on a bacterial lawn on an agar plate). When they get hungry they group together,

demonstrating their allegiance to second-messenger theories of cell biology by emitting pulses of cyclic AMP as an attractant. The aggregate forms a 'slug' that creeps about, its cells differentiating into pre-stalk and pre-spore lineages in preparation for some acrobatics that bring the spore mass to the top of a long vertical anchored stalk.

Bonner observed the rest of the animal kingdom from this little offshore island of slime moulds, a position that gave him a

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Spore tower of the slime mould *Dictyostelium discoideum* (SEM, $\times \sim 150$).

telescopic perspective on all other organisms — from there, there isn't much parallax between cuttlefish and mammals. He could see new questions ("Why reduce down to one cell to cross generations?") that led to great truths about life cycles, and about reproduction generally. He tackled the evolution of complexity in animals, the evolution of culture, the nature of individuality.

Now emeritus professor at Princeton University, Bonner has documented his

intellectual journey among the large biological questions. He describes his progression from the initial choosing of slime moulds, through some cogitations on size, generation time, *Volvox* and life cycles to questions of multicellularity. Then he tackles the big question of development, that great invention so alien to the bacterium or the amoeba, which simply grow, divide, grow, divide. Development of the egg leads to a different organism, the embryo; and this in turn develops through a variety of forms to the breeding stage, which again produces — not more of itself, but eggs. We are so familiar with this that we don't see it as strange; it takes the slime-mould perspective to see that the ability to become larger by developing is not just 'usual', it is also special and remarkable.

We hear about Bonner's working for E. G. Conklin one summer, when they discussed Hans Driesch, and about the great embryologists E. B. Wilson and Conrad Waddington. An account of what Bonner calls "gene nets", which have also been called co-adapted gene complexes and whose results include canalization, comes at the end of a brief paean to DNA, today mandatory in any biology book. Here too is the story of how he was scolded by Haldane for making jokes in a lecture in England (a lesson I never learned, to the dismay of my colleagues). There are asides about many other people, about progress in evolution, and about the Baldwin effect: selection for potential, for the ability to develop a character instead of selection for an overt developmental trait. Unfortunately, he chooses a fantastic example — tameness genes — instead of a real one. There are chapters on getting larger during evolution, on becoming aware and on becoming social. The last, short chapter is about the passage of culture across the generations, about bowerbirds and bluetits, and about writing and computers.

This is a relaxed book, a quiet informative series of chats from the veteran professor who's been there, done that, thought it would be interesting to have a look at one particular thing, discovered another. Perhaps this way of life can survive only in the few old universities content to maintain the ancient traditions of scholarship while the rest of us are reducing everything to job security and the quick buck and losing our academic cultural roots. When we read this book, we can pretend that we have the calm thoughts, the vast knowledge and the discipline of thought that enable Bonner to ask, and to answer, big questions in biology. Then we can go back to the grant application. □

Jack Cohen is in the Ecosystem Analysis and Management Group, University of Warwick, Coventry CV4 7AL, UK.

Limits of growth

Basia Zaba

Living Within Limits: Ecology, Economics, and Population Taboos. By Garrett Hardin. Oxford University Press: 1993. Pp. 339. \$25.

Population Politics: The Choices That Shape our Future. By Virginia Abernethy. Plenum: 1993. Pp. 350. \$26.50.

It has become received wisdom that natural scientists (especially biologists) are extremely alarmed by human population growth, whereas social scientists (especially economists) view it with equanimity. Demographers, who belong to one of the few bridging disciplines, are reviled by both sides in this increasingly polarized debate. These two volumes, the first by an eminent biologist, the other by a professor of psychiatry and anthropology, weigh in unflinchingly on the side of those who blame population growth for most environmental problems. The arguments are not new: Hardin has presented many of them in previous essays and books, and Abernethy in her editorial statements in the journal *Population and Environment*. What is new and prominent in both books is the ruthless implication construed by the authors for US foreign aid and immigration policy.

Hardin's analysis draws on theoretical population biology and thermodynamics: population growth can be positively exponential only for short segments of time, until the carrying capacity imposed by natural resources and the current technological ceiling is approached. The technological ceiling we are now approaching is the ultimate one, because space colonization is impossible and nuclear power too dangerous. Because fast-breeding subgroups will tend to become dominant in any population (whether propensity to breed fast is genetically or culturally transmitted), birth control cannot work on a voluntary basis in the long term, and must therefore be enforced to control population growth.

Abernethy's arguments run something like this: developing countries are not following the same demographic transition pathway experienced by developed nations in the past, because contact with 'Western' culture has eroded traditional forms of fertility control that kept population in balance with natural resources, and foreign aid fosters a 'feel-good factor' that encourages feckless procreation while suppressing mortality levels, which might otherwise moderate population growth. Marxist influences, on the other hand, have led to misguided redistributive policies that have triggered bouts of unsustainable use of 'commonized' resources.



SHOOTING TO KILL — this African elephant was shot in the Selous Game Reserve, Tanzania, for touching a tent rope. It is an example of the needless slaughter that Iain and Oria Douglas-Hamilton rail against in *Battle for the Elephants*. What emerges clearly in this complex and bloody tale of their 20-year crusade to save the elephant is the bitter clash of ideas about how best to deal with the ivory trade. The authors, however, are resolute in their belief that "any trade in ivory opens a Pandora's box of vested interests and commercial incentives" that can never be controlled. Published in paperback by Doubleday on 4 November, £9.99.

Policy conclusions from both analyses are that rapid population growth in less-developed countries will result either in environmental collapse bringing in its wake population collapse, or (at best) in a levelling out of population numbers at a very low standard of living. To limit the number of people enduring squalor or population collapse, and to isolate itself from shared misery, the United States should ban immigration (except possibly from countries that have achieved replacement-level fertility) and abandon current bilingual education programmes which further the ideals of multiculturalism (and by implication help to keep immigrant fertility higher than that of native Americans). It should restrict its foreign aid to the provision of technical know-how, particularly to information about birth control, and to 'micro-loans' administered by non-governmental organizations to further women's small-scale enterprise. Redistributive policies in poor countries should also be discouraged.

Both books are aimed at a general, nonscientific audience. Abernethy's book in particular, with its sloganizing and liberal use of anecdote, will probably irritate serious scholars interested in the critical problems of population, environment and development. Are these works a fair introduction to the subject? I think even those of us who are very concerned about the effects of rapid population growth would have to say no. Neither book deals at all satisfactorily with the problems caused by high per capita consumption. It is hard to imagine a better contemporary illustration of Hardin's

famous *Tragedy of the Commons* than the growth in per capita use of private transport — and yet Hardin himself repeatedly lays problems such as traffic jams and urban smog at the door of population growth. But he does allude to problems of increasing expectations — under the heading of "longages" — and does not exempt more-developed countries, even if he does not believe in global redistribution of wealth. Abernethy seems to believe that only the growing aspirations of growing populations are dangerous: she asserts that it is a right of native-born Americans to have growth in their real wages, and decries the threat to this growth posed by the immigration of cheap labour.

It is highly regrettable that books addressed to a nonspecialist audience should contain logical errors and incoherent definitions of widely used technical terms. Both books suffer from this. Hardin, for example, incorrectly states that exponential growth in per capita resource use in an exponentially growing population produces doubly exponential (Gompertz) growth in resource use, as opposed to exponential growth at the sum of the constituent rates; elsewhere, he explains that the net reproduction rate allows for sterility, whereas it is only pre-reproductive mortality that is taken into account. But at least such mistakes do not affect the thrust of his arguments, and when he has no data to back up his contentions, he proposes "thought experiments" to make his point. Abernethy is less scrupulous: her use of data is highly selective, and in some places contradictory. For example, she quotes from the

demographic literature describing the widely observed phenomenon of moderate increases in fertility preceding long-term fertility declines, and claims that such fertility increase is the principal driving force of population growth in the periods of historical and contemporary demographic transition. This is to ignore conclusions in the very sources she quotes that mortality decline has been far more important in the long run. She criticizes the widely held belief that female education is a cause of fertility decline, pointing out that it is based on correlational evidence, and claims that female paid employment is the most important factor. She does not admit that the evidence for this is also correlational, much less widely observed, statistically weaker and harder to interpret because of timing and selection effects.

These books raise some of the difficult questions about present equity and future welfare that cannot be ignored, even by those who profoundly disagree with the authors' policy conclusions. But there are many other distributional issues that are important in population-environment relationships, quite outside the blame allocation debate. If 'luxury' consumption is reduced, how can current economic systems be adapted to cope with this and still generate enough surplus to be invested in environmental protection measures, including effective birth-control programmes? If certain kinds of industrial activity have to be curtailed, how can this be done most equitably with respect to employment, so that the largest possible fraction of the population can still afford to purchase essential products? Neither Hardin nor Abernethy considers these important issues.

Both authors concentrate on the threat that population growth in less-developed countries poses to the standard of living in the United States, whereas the threats to the local environments and social fabric of countries actually experiencing these growth rates are of a far higher order of magnitude. By contrast, the global consequences of luxurious levels of consumption in more-developed countries, such as atmospheric warming, are likely to be much more severely felt in less-developed countries. Elitist calls for isolationist measures are a godsend to those still reluctant to face the real problems of population growth, and are likely only to polarize the debate in sterile ways. How fortunate that there are economists, biologists and demographers engaged in a more productive dialogue about problems that can best be faced jointly by developed and developing countries. □

Basia Zaba is in the Centre for Population Studies, London School of Hygiene and Tropical Medicine, 99 Gower Street, London WC1E 6AZ, UK.

Video cytokine

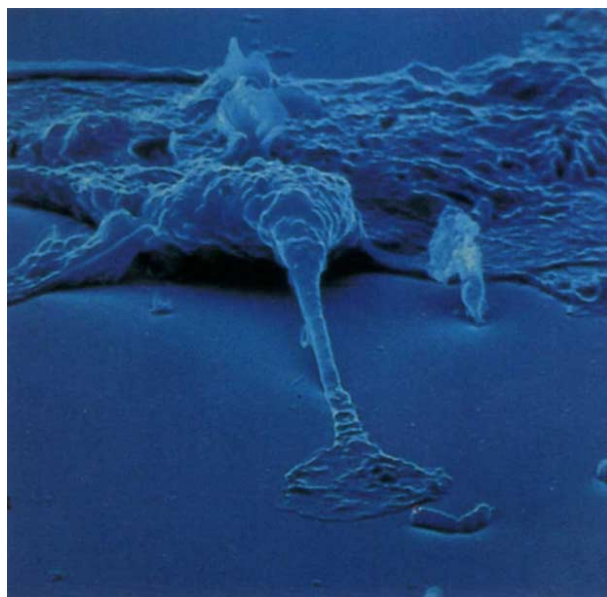
F. Y. Liew

Interferon Gamma: The Immune Interferon. A video produced by Cambridge Scientific Films on behalf of Boehringer Ingelheim. VHS PAL colour; running time, 23 minutes; distribution details available on application to Boehringer Ingelheim.

RESEARCH laboratories rarely indulge in the art of video, but when they do, the result can be as informative and colourful as any good seminar. An unusual collaboration between the pharmaceutical company Boehringer Ingelheim and Dr Greg Bancroft and his team at the London School of Hygiene and Tropical Medicine has now produced this highly instructive

antigen presentation, modulates immunoglobulin and cytokine synthesis, increases the microbicidal and tumoricidal activity of macrophages, promotes the differentiation of a number of myeloid cell lines and inhibits cell growth. It seems also that the increased production of interferon gamma during infections can promote many diseases. For example, interferon gamma is essential for the induction of type 1 T helper cells and for the inhibition of proliferation of type 2 T helper cells, cells that help in the synthesis of immunoglobulin E, a mediator of hypersensitivity reactions in allergic diseases. Interferon gamma is also produced by natural killer cells, which can kill cells in the absence of antigenic stimulation, a finding made by Bancroft and his colleagues. These are just a few of a host of functions of this versatile cytokine.

By combining excellent narration and



Fatal attraction — a macrophage prepares to engulf a bacterium.

film about interferon gamma and its important role in immunity against infection. The programme is replete with Lennart Nilsson's arresting electron micrographs, an example of which is shown here.

Since the discovery of interferons by Alec Isaacs and Jean Lindenmann in 1957, great hope has been held out for their use as therapeutic agents. Alas, the promise of this diverse family of glycoproteins has not yet been fulfilled. Secreted by virus-infected cells, they protect noninfected cells from infection by inducing host cell enzymes that affect the transcription and translation of viral genes. They also have immunoregulatory functions, interferon gamma in particular.

Interferon gamma — dubbed the 'immune interferon' — is secreted mainly by antigen-activated T cells in inflammatory and autoimmune diseases. In addition to its antiviral effects, this interferon induces the expression of class I and II major histocompatibility molecules involved in

photography, this video succeeds in presenting a great deal of detailed information clearly. The package is intended primarily as an educational aid for clinicians, but it should also prove to be a useful revision aid for undergraduates. Further information about the mechanism of induction of interferon gamma and its role in the regulation of type 2 T helper cells would capture the interest of a more advanced audience; and the latest results on the clinical trials using interferon gamma to treat hepatitis, leishmaniasis and leprosy would make the programme even more appealing to clinicians. But these are mere quibbles in the face of the major achievement — the video's elegant and concise presentation of so much complex information, a feat so rarely seen in television science programmes. □

F. Y. Liew is in the Department of Immunology, University of Glasgow, Glasgow G11 6NT, UK.

New roles for G-protein $\beta\gamma$ -dimers in transmembrane signalling

David E. Clapham* & Eva J. Neer†

When a membrane-bound receptor acts on a G protein, the GTP-binding or G_α subunit dissociates from the $G_{\beta\gamma}$ dimer. Until recently, the G_α subunit alone was thought to act on the enzymes and ion channels controlled by these proteins. Newer evidence indicates that the $G_{\beta\gamma}$ dimer also plays a major part in signal transmission, enhancing the complexity of the possible interactions between the G proteins and their targets.

ONCE an external signal has been received by a membrane-bound receptor, it must be passed to the appropriate effector molecules inside the cell. Hundreds of receptors, most with seven transmembrane α -helices, signal to cellular enzymes and ion channels through G proteins. These proteins become active on binding GTP but their intrinsic ability to hydrolyse GTP eventually converts them to the inactive GDP-bound form. They remain in this state until the GDP is exchanged for GTP¹⁻⁵ in response to a receptor (see Fig. 1 for the regulatory cycle). G proteins contain three different subunits, G_α , G_β and G_γ , only the first of which actually binds and hydrolyses GTP. Binding of GTP or a non-hydrolysable analogue such as GTP- γ S activates the G_α subunit and, at least in solution, causes G_α to dissociate from $G_{\beta\gamma}$ (G_β does not dissociate from G_γ). Free G_α subunits were initially shown to activate two effectors (adenylyl cyclase and retinal cyclic GMP phosphodiesterase), and it therefore seemed reasonable to assume that G_α was the subunit that directly controlled the activity of all effectors. $G_{\beta\gamma}$ was thought to reverse the action of G_α by reforming the inactive heterotrimer and guiding G_α back to the receptor for reactivation. This view, which shaped ideas about G-protein function for more than a decade, was challenged in 1987 by a report of an effector activated by $G_{\beta\gamma}$ (ref. 6). Since then, the list of $G_{\beta\gamma}$ targets has grown steadily until now as many effectors are known to be regulated by this subunit as by G_α .

Activated G proteins thus seem to produce a bifurcating signal allowing for both subtle regulation and complex feedback control of receptors and effectors. For instance, effectors can be regulated by G_α only, by $G_{\beta\gamma}$ only, by G_α or $G_{\beta\gamma}$ (that is, both subunits can regulate the effector) or by G_α and $G_{\beta\gamma}$ together (that is, regulation by one subunit depends on prior priming by the other). The two signalling molecules involved can produce divergent signals if each molecule regulates a different effector, or convergent ones if both molecules act on the same target. Depending on the effector, the G-protein subunits can activate, inhibit or antagonize each other. The predominant effect will depend on the exact cellular complement of receptors, G proteins and effectors. Too little is known for a complete description of the interactions underlying any one signalling event; here we concentrate instead on the evidence that $G_{\beta\gamma}$ interacts directly with effectors and its implications for our understanding of G-protein action.

Structure of the $G_{\beta\gamma}$ subunit

The four known mammalian $G_{\beta\gamma}$ subunits, each about 340 amino acids long, share more than 80% amino-acid identity. The amino-acid differences are scattered throughout the sequence^{4,7,8}. Despite this close resemblance, they cannot form dimers with each kind of G_γ subunit; $G_{\beta 1}$ can associate with $G_{\gamma 1}$ or $G_{\gamma 2}$, for example, whereas $G_{\beta 2}$ can associate with $G_{\gamma 2}$ but not with $G_{\gamma 1}$, and $G_{\beta 3}$ is unable to associate with either $G_{\gamma 1}$ or $G_{\gamma 2}$ (refs 9, 10). The amino-acid sequence of G_β suggests it contains two

types of structure. The amino-terminal region is predicted to form an amphipathic α -helix which could participate in coiled-coil interactions¹¹. The remainder of the protein consists of seven repeating units, each about 43 amino acids long¹². Similar repeating units occur in several other proteins whose functions seem to be unrelated to signal transduction¹³. We do not know exactly what features of the repeating structure necessitate its conservation among so many different proteins.

At present, seven different G_γ subunits are known^{4,14}. They are about 75 amino acids long and are much more heterogeneous than the G_β subunits. Retinal $G_{\gamma 1}$ is only 38% identical to brain $G_{\gamma 2}$ and equally different from other mammalian G_γ subunits. The G_γ subunits also differ in their modification by prenyl groups: $G_{\gamma 1}$ is farnesylated, whereas $G_{\gamma 2}$ is geranyl geranylated (reviewed in ref. 15). If prenylation is blocked by mutation of the carboxy-terminal cysteine which is the site of modification, $G_{\beta\gamma}$ dimers can still form, but they do not attach to cell membranes^{16,17}, stimulate adenylyl cyclase or interact with some G_α subunits¹⁸. The G_γ subunit is crucial for determining the function of the $G_{\beta\gamma}$ subunits. The function of $G_{\beta 1\gamma 1}$ (found in the retina) is very different from that of $G_{\beta 1\gamma 2}$ (found in the brain and elsewhere). As the G_β subunits are the same, functional differences between the two $G_{\beta\gamma}$ forms are likely to be due to the different G_γ subunits. Several examples of these differences will be given below.

Activation of $G_{\beta\gamma}$ subunits

G proteins can be persistently activated by binding non-hydrolysable GTP analogues, such as GTP- γ S, to the G_α subunit. In solution, activation by GTP- γ S clearly dissociates G_α from $G_{\beta\gamma}$, but it is not certain whether dissociation is essential for $G_{\beta\gamma}$ activation and whether the subunits separate in the membrane or simply change conformation without physical separation. Yi *et al.*¹⁹ showed that G_α could assume a conformation resembling the activated state while chemically crosslinked to $\beta\gamma$, and Matterna *et al.*²⁰ described an activated but undissociated state. There is also evidence for subunit exchange in the membrane²¹, however, although the range of mobility available to these proteins is unclear. Whether or not they separate, however, both G_α and $G_{\beta\gamma}$ remain active as long as GTP is bound to G_α . When GTP is hydrolysed to GDP, G_α is not only inactivated itself, but also inactivates $G_{\beta\gamma}$. Activation of $G_{\beta\gamma}$ can thus be blocked by interruption of normal G_α function. For example, pertussis toxin ADP-ribosylates the G_α subunit of one class of GTP-binding proteins¹⁻⁵ leaving the G_α subunit unable to interact with a receptor or to exchange GDP for GTP, and locking both $G_{\beta\gamma}$ and G_α in their inactive states. $G_{\beta\gamma}$ increases G_α 's affinity for GDP by about 100-fold, stabilizing the inactive state²². The activity of both subunits may also be regulated by other proteins that bind only one of the subunits, such as calmodulin²³ and phosducin²⁴, which act on $G_{\beta\gamma}$, or the neural protein GAP 43, which acts on one form of G_α (ref. 25).

G_{βγ} activates many effectors

Table 1 summarizes the effectors known to be regulated by G_{βγ}. Regulation by both G_α and G_{βγ} subunits has increasingly come to seem the rule rather than the exception.

K⁺ channel (I_{K,ACH}). The first indication that G_{βγ} subunits could control effectors directly came with the report in 1987 that G_{βγ} purified from bovine brain activated the cardiac K⁺ channel (I_{K,ACH}) normally regulated by the muscarinic acetylcholine receptor. Using patch clamp techniques, the intracellular face of the patch can be perfused with either a GTP analogue or purified G_{βγ} or G_α subunits, greatly increasing the frequency with which the K⁺ channels open^{6,26}. Activation of I_{K,ACH} by either subunit does not seem to be conditional on prior activation by the other; either G_α or G_{βγ} can activate the channel by itself²⁷. Elegant recent experiments by Ito *et al.*²⁸ show that G_{βγ} activates I_{K,ACH} more effectively than G_α, whereas G_α alone can activate another type of K⁺ channel, I_{K,ATP}, in the same patch. G_α activates I_{K,ACH} at lower concentrations (~10 pM rather than ~10 nM) but G_{βγ} activates 95% of patches whereas G_α only activates ~30% of patches^{27,29}. The concentration of G_{βγ} that activates the K⁺ channel, however, is similar to the concentration shown to regulate other effectors.

Without purified I_{K,ACH}-channel protein, it is still unclear whether G_α or G_{βγ} activate the channel directly or via intermediates. Cell patches are large areas of membrane (1–10 μm²) containing few channels but many other proteins and membrane-associated second messengers. Kim *et al.*³⁰ suggested that G_{βγ} activated I_{K,ACH} via phospholipase A₂ (PLA₂) because an antibody shown to block PLA₂ activity in other systems blocked G_{βγ} activation of I_{K,ACH}. Although arachidonic acid and its metabolites activate the channel, they do not do so as rapidly or as well as G_{βγ} (refs 30, 31) and it is not certain whether G_{βγ} activates I_{K,ACH} directly (ref. 6) or indirectly via PLA₂ (ref. 30). Our current hypothesis is that its action is direct and that arachidonic acid modulates channel activity.

Phospholipase A₂. G_{βγ} subunits increase arachidonic acid production by PLA₂ in G-protein-depleted retinal membranes reconstituted with purified retinal G_{βγ} (ref. 32). The concentration of G_{βγ} needed is high (0.6 μM), but the G_{βγ} concentration is estimated to approach 500 μM in the rod outer segments. These findings have not been pursued, except for the suggestion mentioned above that G_{βγ} activates I_{K,ACH} via PLA₂ (ref. 30). PLA₂ can also be activated by G_α subunits, but little is known about the effects of G_α and G_{βγ} on purified PLA₂.

Yeast mating response. Strong evidence that G_{βγ} is important in transducing signals from a cell-surface receptor comes from genetic analysis of the yeast mating pheromone pathway. Null mutations in the yeast G_β and G_γ homologues STE4 and STE18 block the mating and pheromone responses. In contrast, disruption of the yeast G_α homologue SCG1 (GPA1) leads to a constitutive response. These findings have been interpreted to mean that the yeast G_α homologue acts as a negative regulator of G_{βγ}, and that G_{βγ} constitutes the direct signalling element³³. The target of G_{βγ} may be the protein kinase Ste20 (ref. 34).

Adenylyl cyclase. Adenylyl cyclase is one of the best studied examples of an effector regulated by a G_α subunit, G_{as}. Cyclic adenosine monophosphate (cAMP) production can be stimulated by pure GTP-γS-bound G_{as} and the activation is reversed by excess G_{βγ}, presumably by tying up G_{as} in an inactive heterotrimer^{1,5}. Inactivation of G_{as} by G_{βγ} may be one mechanism for hormonal inhibition of adenylyl cyclase, although other G_α subunits may also directly inactivate the enzyme^{35,69}.

A more detailed picture appeared when four isoforms of adenylyl cyclase were cloned and expressed in Sf9 insect cells along with recombinant individual G-protein subunits. Type I adenylyl cyclase, the calmodulin-sensitive enzyme from the central nervous system, is activated by GTP-γS-bound G_{as} and inhibited by G_{βγ}. Types II and IV adenylyl cyclase are structural homologues of type I, but are insensitive to calmodulin and regulated very differently. They are modestly activated by G_{as},

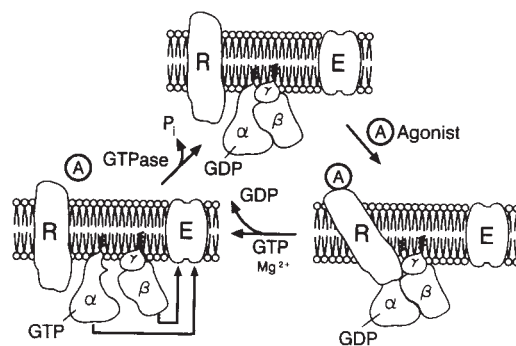


FIG. 1 Both subunits of the GDP-bound G-protein heterotrimer interact with the receptor. Agonist-bound receptor catalyses the exchange of GTP for GDP on the G_α subunit. GTP-liganded G_α presumably dissociates from G_{βγ} and each subunit is free to activate other proteins. The duration of subunit separation is timed by the rate of G_α-mediated hydrolysis of GTP. Some effectors are involved in determining the duration of their own activation because they increase the rate of GTP hydrolysis of G_α.

but synergistically activated five- to sixfold further by G_{βγ} (ref. 36). The solubilized and partially purified type II enzyme retains its susceptibility to stimulation by G_{βγ}, suggesting that this is an intrinsic property³⁷. The activation of adenylyl cyclase types II and IV is an example of conditional activation, because activation by G_{βγ} requires that the enzyme be primed by G_{as}. Experiments with chimaeric adenylyl cyclases indicate that the site at which G_{βγ} acts is in the C-terminal half of the molecule. G_{βγ} neither activates nor inhibits type III adenylyl cyclase³⁶. Different G_{βγ} isoforms can activate type II adenylyl cyclase to different extents, transducin G_{βγ} (G_{β1γ1}) being about one-tenth as effective as G_{β1γ2}, G_{β2γ2} or G_{β2γ3}. G_{γ2} subunits mutated at the site of prenylation (C68S) are able to form G_{βγ} dimers, but these dimers cannot activate or inhibit adenylyl cyclase¹⁸.

G_{βγ} subunits are liberated by stimulation of many kinds of receptors, including those coupled to G proteins whose normal function is to inhibit adenylyl cyclase (the G_{ai} group). Such inhibitory receptors should activate type II adenylyl cyclase if GTP-bound G_{as} is also present. These circumstances were created by transfecting HEK293 and COS7 cells with expression plasmids encoding a constitutively active mutant G_{as}, type II adenylyl cyclase and the α₂-adrenergic, dopamine or adenosine receptors (all G_{ai}-linked). Under these conditions, the inhibitory receptors were indeed able to stimulate type II adenylyl cyclase³⁸, presumably acting through G_{βγ}.

Phospholipase C. Phospholipase C (PLC) exists in several isoforms, some of which (such as PLCβ1–4) are regulated by G proteins whereas others (such as PLCγ) are not^{39,40}. PLCβ isoforms 1–3 are regulated both by G_α subunits of the G_q class (G_{αq/11}, G_{α16}) and by G_{βγ} subunits^{41–46}. PLCβ4 is not regulated by G_{βγ} (S. C. Rhee, personal communication). Analysis of diacylglycerol production by single PLCβ isoforms expressed in transfected cells or purified from several tissues showed that G_{βγ} stimulates PLCβ3 most and PLCβ1 least, whereas G_{αq/11} subunits stimulate PLCβ1 most and PLCβ3 least^{47–49}. Activation of PLCβ isoforms by G_{αq/11} and G_{βγ} is independent and not conditional on priming by either subunit. Variable patterns of stimulation occur when both subunits are present simultaneously⁴⁹. The independent actions of the subunits reflect the different sites on the PLCβ molecule to which they bind, with G_{βγ} binding to the N-terminal and G_α binding to the C-terminal region⁷⁰ (P. Gierschik, S. G. Rhee and J. Exton, personal communication). Muscarinic receptors expressed in COS cells can activate PLCβ2 as long as G_{βγ} is simultaneously transfected, indicating that receptors can initiate signalling cascades working through G_{βγ} (ref. 48).

The recent finding that PLC β increases the GTPase activity of the G_α subunit to which it binds indicates that an effector can exert negative feedback on a G-protein signalling subunit⁵⁰. Another effector that increases the GTPase of its activating G_α subunit is the retinal cyclic GMP phosphodiesterase⁵¹. These activities are similar to those of GTPase activating proteins (GAPs), which enhance GTP hydrolysis by a family of low M_r G proteins that include p21^{ras} (ref. 52). It will be interesting to see whether $G_{\beta\gamma}$ can modulate the GTPase-activating (GAP) function of PLC β . For example, inhibition of effector GAP function by $G_{\beta\gamma}$ might lead to synergism between G_α and $G_{\beta\gamma}$. This would not explain the synergism of G_α s and $G_{\beta\gamma}$ in activating type II adenylyl cyclase, however, as that synergism was observed using the nonhydrolysable GTP analogue GTP- γ S. Because the GTPase activity of G_α determines the time for which both G_α and $G_{\beta\gamma}$ are active, regulation of effector GAP activity by either subunit would affect the function of both.

Calcium channels. Ca²⁺ influx mediated by 'N-type' (high voltage-activated, ω -conotoxin-sensitive, slowly inactivating) Ca²⁺ channels can be inhibited by the muscarinic acetylcholine receptor, the α -adrenergic receptor and somatostatin receptor, acting via subtypes of $G_{\alpha o}$, whereas Ca²⁺ influx mediated by L-type (high voltage-activated, dihydropyridine-sensitive, persistent-current) channels can be stimulated by β -adrenergic receptors acting via $G_{\alpha s}$ (refs 53, 54). Whether $G_{\alpha s}$ interacts directly with L-type channels or entirely indirectly through phosphorylation is controversial⁵⁵, and the mechanism by which $G_{\alpha o}$ inhibits N-type channels is unknown⁵⁴. Regardless of the effector mechanism, an inhibitory pathway shows remarkable G-protein subtype specificity. Nuclear microinjection of antisense oligonucleotides into GH3 cells shows that the somatostatin receptor inhibits a Ca²⁺ channel via $G_{\alpha o2}\beta_1\gamma_3$, whereas the M4 muscarinic receptor inhibits the same channel by $G_{\alpha o1}\beta_3\gamma_4$ (refs 56–58). Because elimination of either of the appropriate G_α or $G_{\beta\gamma}$ completely blocks the Ca²⁺-channel inhibition, the two subunits may each independently regulate the channel. Alternatively, different $G_{\beta\gamma}$ subunits may specify which receptor activates which $G_{\alpha o}$ subtype, or each $G_{\alpha o}$ may have a strict preference for a particular $G_{\beta\gamma}$. Experiments in intact cells cannot sort out these possibilities. Whichever is correct, these experiments provide dramatic examples of cellular specificity of subunit function.

Receptor kinases. $G_{\beta\gamma}$ has long been known to regulate receptor function directly by enhancing receptor interaction with G_α GDP; receptors bind the heterotrimer more effectively than they bind dissociated G_α subunits^{59,60}. The $G_{\beta\gamma}$ subunit may itself bind to receptors, and indeed co-purifies with the β -adrenergic receptor⁶¹. $G_{\beta\gamma}$ may also modulate receptor function by controlling the location of receptor-specific protein kinases that blunt receptor activity. $G_{\beta\gamma}$ has recently been found to produce a 10-fold increase in the agonist-dependent phosphorylation of purified muscarinic and β_2 -adrenergic receptors by protein kinases of the β -adrenergic receptor (β ARK) family^{62,64}.

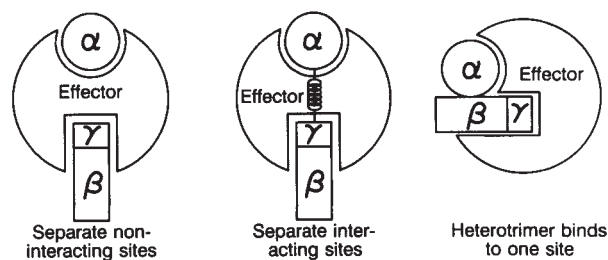


FIG. 2 Some possibilities for G-protein subunit-effector interactions. a, G_α and $G_{\beta\gamma}$ may bind individually to separate non-interacting sites; b, G_α and $G_{\beta\gamma}$ may bind individually to separate interacting sites; c, G_α and $G_{\beta\gamma}$ may bind as an activated heterotrimer to a single site.

TABLE 1 Effectors regulated by G-protein subunits

Effector	Regulator		Type of $G_\alpha/G_{\beta\gamma}$ interaction	Ref.
	G_α	$G_{\beta\gamma}$		
K ⁺ channel ($I_{K,ACH}$)	Yes	Yes	Independent	6, 26–31
K ⁺ channel ($I_{K,ATP}$)	Yes	No		28
PLA ₂	?	Yes		32
Pheromone response	No	Yes	Antagonistic	33
Adenylyl cyclase I	Yes	Yes		36
Adenylyl cyclase II(IV)	Yes	Yes		36
Adenylyl cyclase III	Yes	No	Independent	36
PLC β 1–3	Yes	Yes		44–48
Ca ²⁺ channels (L and N)	Yes	Unknown		53–55
cGMP PDE	Yes	No		71
Muscarinic receptor kinase*	No	Yes		62
β ARK*	No	Yes		63, 64

Effectors shown to be regulated by one or both G-protein subunits. 'Yes' indicates an interaction, whether stimulatory or inhibitory to effector function; 'No' indicates no demonstrated effect on function of the effector.

* Regulation of these kinases is probably through their translocation by $G_{\beta\gamma}$ from the cytosol to their membrane-bound targets.

Rather than increasing the intrinsic activity of β ARK, $G_{\beta\gamma}$ seems to move it from the cytosol to the plasma membrane where it can phosphorylate the agonist-bound receptor. It is not clear whether the receptor activates the kinase that has been delivered to it, or whether the receptor is simply a substrate for β ARK, although the activation of rhodopsin kinase (which is related to β ARK) by photo-rhodopsin indicates that the former possibility is likely⁶⁵. The finding that $G_{\beta\gamma}$ can regulate receptors by controlling their phosphorylation and subsequent desensitization gives new insight into complex feedback mechanisms for fine-tuning hormone responses.

Modulators of $G_{\beta\gamma}$ function

Phosducin. Phosducin is a retinal phosphoprotein of unknown physiological function²⁴. It forms a specific complex with retinal $G_{\beta\gamma}$ ($\beta_1\gamma_1$) and modulates the ability of $G_{\beta\gamma}$ to interact with retinal G_α (ref. 24). *In vitro*, phosducin inhibits the retinal cyclic GMP phosphodiesterase, probably by binding to $G_{\beta\gamma}$, and preventing the heterotrimer from associating with activated rhodopsin. It may therefore modulate the visual pathway by titrating $G_{\beta\gamma}$. It is not known whether phosducin has any enzymatic activity of its own. The $\beta\gamma$ subunit may also bind calmodulin, but the physiological role of this interaction has not been defined²³.

The mechanism of $G_{\beta\gamma}$ action

The current working model of G-protein action must be altered to accommodate $G_{\beta\gamma}$'s ability to regulate receptor function both directly and indirectly and to control some effectors directly.

Regulation of receptors. The $G_{\beta\gamma}$ subunit can regulate receptor function by enhancing the receptor's ability to interact with G_α or by bringing a receptor-specific kinase to the receptor to desensitize it. In principle, a given $G_{\beta\gamma}$ subunit could be very effective at modulating the interaction of G_α with the receptor, but poor at bringing the appropriate kinase to it or the reverse, creating considerable flexibility in the extent to which a particular G_α – $G_{\beta\gamma}$ pair could be activated by a receptor.

The mechanism by which $G_{\beta\gamma}$ enhances receptor phosphorylation may be the first clue to the general function of $G_{\beta\gamma}$, that of bringing proteins to receptors for agonist-dependent regulation. $G_{\beta\gamma}$ performs this role for both G_α and β ARK, but many

other regulatory enzymes may be targeted to the membrane-bound signalling components by specific $G_{\beta\gamma}$ pairs. The ability of $G_{\beta\gamma}$ to promote macromolecular assembly suggests that $G_{\beta\gamma}$ may play a similar part in the regulation of intracellular vesicular traffic by heterotrimeric G proteins⁶⁶.

Regulation of effectors. It has been puzzling that effector activation of G-protein subunits requires substantially higher concentrations of $G_{\beta\gamma}$ than GTP- γ S-bound G_{α} and this difference has been used to argue that activation by G_{α} is direct^{26,67}. Recently, Gilman *et al.*^{68,69} have pointed out that such a difference is to be expected. In the cell, G_{α} subunits are not irreversibly activated by GTP- γ S, but are activated by GTP that is constantly being cleaved to GDP in a process enhanced by some effectors. Thus, it should take less of the permanently activated form of G_{α} than of the transiently activated form to stimulate an effector. A physiologically meaningful dose-response comparison would be between G_{α} -GTP and $G_{\beta\gamma}$, but this is technically very difficult. If the hypothesis is correct, the amount of G_{α} GTP and $G_{\beta\gamma}$ needed to stimulate an effector would be roughly equal and difference between the levels of G_{α} GTP- γ S and G_{α} GTP needed to activate an effector would depend on the intrinsic rate of hydrolysis and on the GTPase-activating (GAP) capacity of the effector. This suggests that $I_{K,ACH}$ should have a brisk GAP activity.

It is now clear that the effector subtype critically determines which G-protein subunit or combination of subunits is active. For example, types II and IV (but not types I or III) adenylyl cyclases are activated by $G_{\beta\gamma}$ subunits. $PLC\beta_2$ is activated more by $G_{\beta\gamma}$ than is $PLC\beta_1$. $G_{\beta\gamma}$ subunits translocate the β ARK family but not rhodopsin kinase⁶⁴, and G_{α} activates $I_{K,ATP}$, while $G_{\beta\gamma}$ and G_{α} both activate $I_{K,ACH}$ (refs 6, 26–28).

As noted above, there are in principle five different ways in which G_{α} and $G_{\beta\gamma}$ together can modulate any given effector.

Although in no case is the molecular basis fully understood, examples of each of these ways are now known. The existence of effectors controlled by G_{α} subunits alone (for example, adenylyl cyclase type III), by $G_{\beta\gamma}$ subunits alone (such as the yeast mating response), by both subunits acting independently (the muscarine-gated cardiac K^+ channel $I_{K,ACH}$ and $PLC\beta$ isoforms), by both subunits acting synergistically (adenylyl cyclase types II and IV) and by both subunits acting antagonistically (adenylyl cyclase type I) indicates that the possibilities inherent in a bifurcating signalling system have been exploited to a degree that could not have been imagined a few years ago.

Each of these modes of activation suggests a different molecular mechanism (Fig. 2). For example, different effector isoforms may have binding sites that specifically recognize one or the other subunit. When both subunits bind, these sites could be independent or interacting. Binding of one subunit (for example, G_{α}) to its site might make binding of other subunits to the target enzyme more or less effective. Because activated heterotrimers may exist transiently^{19,20}, it is possible that the heterotrimer may interact with the effector and even be stabilized by it. Detailed kinetic analysis of the subunit actions will answer some of these questions.

Although the idea initially seemed quite heretical⁶⁷, it is now clear that both G-protein subunits interact with effectors and receptors and have an active role in signal transduction. The protein-protein interactions that coordinate these responses are only beginning to be understood, and new experiments are sure to produce even more surprises. □

David E. Clapham is at the Department of Pharmacology, Mayo Foundation, Guggenheim 7, Rochester, Minnesota 55905, USA, and Eva J. Neer is at the Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, 75 Francis Street, Boston, Massachusetts 02115, USA.

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Direct observation of magnetic flux lattice melting and decomposition in the high- T_c superconductor $\text{Bi}_{2.15}\text{Sr}_{1.95}\text{CaCu}_2\text{O}_{8+x}$

R. Cubitt^{*}, E. M. Forgan^{*}, G. Yang^{*}, S. L. Lee[†], D. McK. Paul[‡],
H. A. Mook[§], M. Yethiraj[§], P. H. Kes^{||}, T.W. Li^{||}, A. A. Menovsky[•],
Z. Tarnawski^{||} & K. Mortensen[#]

^{*} School of Physics and Space Research, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

[†] Physik-Institut der Universität Zürich, CH-8057 Zürich, Switzerland

[‡] Department of Physics, University of Warwick, Coventry CV4 7AL, UK

[§] Solid State Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831-6393, USA

^{||} Kamerlingh Onnes Laboratorium, Leiden University, PO Box 9506, 2300 RA, Leiden, The Netherlands

[•] Department of Physics, University of Amsterdam, 1018 XE Amsterdam, The Netherlands

[#] Physics Department, Risø National Laboratory, DK-4000 Roskilde, Denmark

The flux line lattice inside a single crystal of $\text{Bi}_{2.15}\text{Sr}_{1.95}\text{CaCu}_2\text{O}_{8+x}$ has been observed using small-angle neutron diffraction. The diffracted intensity goes rapidly to zero at a magnetic-field-dependent flux lattice melting temperature; this melting coincides with the appearance of finite resistance within the superconducting state. The flux lattice signal can also be made to disappear at low temperatures, by applying a sufficiently high field, probably because of the decomposition of flux lines into two-dimensional ‘pancake’ vortices.

THE discovery of high-transition-temperature (high- T_c) superconductivity above liquid-nitrogen temperature, in various compounds containing CuO_2 planes, has led to hopes of increased applications for superconductivity. The properties of these new materials are, however, different in some ways from conventional superconductors: the increased temperatures involved, the planar crystal structures, and low carrier concentrations all mean that thermal fluctuations are far more important than in low- T_c materials, particularly when a magnetic field, B , is applied¹, so that quantized flux lines are formed. Magnetization and a.c. electrical response measurements show² an ‘irreversibility line’ in the B - T plane: above this line, flux lines move in response to external forces, giving reversible magnetization curves and a finite electrical resistivity, even though the temperature may be well below T_c . In the irreversible region, flux lines are pinned by defects in the superconducting crystal. This irreversibility line, dividing pinned from unpinned flux, has been associated with the melting of a vortex lattice or a vortex ‘glass’^{1,3,4}, although microscopic evidence for this has not been obtained. Indeed, for some of the a.c. measurements, loss peaks have been attributed to changes in a.c. penetration depths⁵, rather than intrinsic changes in the flux lattice.

It has been clear for some time that the response to high magnetic fields of the Bi-based high- T_c superconductors is rather different from that of $\text{YBa}_2\text{Cu}_3\text{O}_{7-x}$ (YBCO). In particular, dissipation peaks in vibrating-reed experiments on $\text{Bi}_{2.15}\text{Sr}_{1.95}\text{CaCu}_2\text{O}_{8+x}$ (BSCCO) have been interpreted as evidence for melting of the flux line lattice well below T_c (ref. 6), and non-zero resistivity is observed down to much lower temperatures in this material⁷. It has become increasingly clear that the difference in behaviour between BSCCO and YBCO is due to the huge anisotropy of the normal and superconducting properties of BSCCO, which increases the instability of the flux lattice, particularly with respect to thermal fluctuations⁴. The highly layered structure of Bi- and Tl-based materials tends to confine supercurrents to the CuO_2 planes, in which currents flow as ‘pancake’ vortices. There are attractive interactions between pancake vortices in different planes, so that they tend to line up,

giving essentially three-dimensional flux line behaviour. But on increasing the flux density to a point where the in-plane repulsion between pancakes overcomes their inter-plane attraction, the presence of pinning can break up any order in the field direction, leading to two-dimensional behaviour. In addition, when thermal fluctuations become large, a melting transition is possible from either of the pinned states previously mentioned.

Here we apply the technique of small-angle neutron scattering, which was earlier used to reveal the spatial arrangement of flux lines in YBCO^{8,9}, to investigate flux line structures in BSCCO. At low fields and temperatures we see a distinct diffraction signal, which disappears on increasing temperature at constant field. We regard the temperature at which the intensity goes to zero as the melting temperature of a three-dimensional flux lattice. Magnetic measurements on a small part of our sample show that the melting occurs very close to the irreversibility line, at which finite electrical resistance appears. At fields above 0.1 T, we detect no diffraction signal from the flux lattice, even at the lowest temperatures. This loss of signal is probably due to the distortion of straight flux lines by pinning of individual pancakes, which is expected to be more evident at high fields. Thus in BSCCO a simple three-dimensional flux lattice occurs only in a small corner of the B - T phase diagram.

Experimental details

In recent muon spin rotation (μSR) measurements¹⁰, there were clear indications of a flux line lattice in BSCCO at fields less than 50 mT and at temperatures below the irreversibility line; our neutron scattering investigation has accordingly been concentrated in this region of the B - T plane. The sample (which was a part of the μSR sample) was a 180-mg plate-like crystal of BSCCO with chemical composition $\text{Bi}_{2.15}\text{Sr}_{1.95}\text{CaCu}_2\text{O}_{8+x}$ prepared as described in ref. 11, with its c -axis parallel (within a degree) to both an applied magnetic field and an incident neutron beam (provided by the cold source at the DR3 reactor, Risø, Denmark). The sample was mounted in a variable-temperature cryostat capable of cooling to 1.5 K. The small-angle neutron scattering instrument allowed a 6-m collimating

path for the beam before the sample, and a 6-m path from the sample to an area detector of 59 cm diameter, with a resolution of 8 mm. At the lowest fields we employed (20 mT), the flux line spacing was greater than 3,000 Å; an incident neutron wavelength λ_n of 19.5 Å (with a wavelength spread, $\delta\lambda_n/\lambda_n=0.18$ full-width half-maximum (FWHM)) was used to give sufficiently large scattering angles. It was necessary to take a 'background' measurement by cooling in zero field, to account for small-angle scattering by the cryostat and sample in the absence of a flux lattice. Observations were made by cooling in a field and subtracting the background counts. Slight movements ($\ll 1$ mm) of the sample or detector between these measurements resulted in imperfect subtractions near the main beam. These effects were removed by mathematically shifting the background by a fraction of a pixel before subtraction.

Melting transition

A typical diffraction pattern after subtraction and smoothing is shown in Fig. 1a. The axis of the beam is nearly parallel to the flux lines, so the diffracted spots are approximately equal in intensity¹². Unlike YBCO under similar conditions⁹, a hexagonal

diffraction pattern is observed. One set of flux lattice planes (that is, pair of diffraction spots) is preferentially aligned with the *a* axis of the crystal, which lies 15° clockwise to the horizontal in Fig. 1, whereas decoration experiments at a somewhat lower field did not show a preferred orientation¹³. After instrumental effects have been removed, however, the tangential width of the spots corresponds to a 35° FWHM variation of the angular orientation of the planes of flux; thus the flux lattice orientation is not fixed to that of the crystal lattice throughout the volume of the sample. The radial width of the spots is mainly controlled by instrumental effects¹⁴; with our present statistics, we are not able to obtain definite information from this about the spread of the flux lattice-parameter.

On increasing the temperature of the sample, the diffracted intensity goes rapidly to zero at a temperature T_m which is much less than the T_c of 85 K. Typical diffraction patterns as a function of temperature are shown in Fig. 1. The results of temperature scans at two different applied fields are shown in Fig. 2. It is clear that T_m decreases with increasing *B*. In Fig. 3, three values obtained for T_m are plotted in the *B*-*T* plane, along with magnetic measurements of the irreversibility line and a

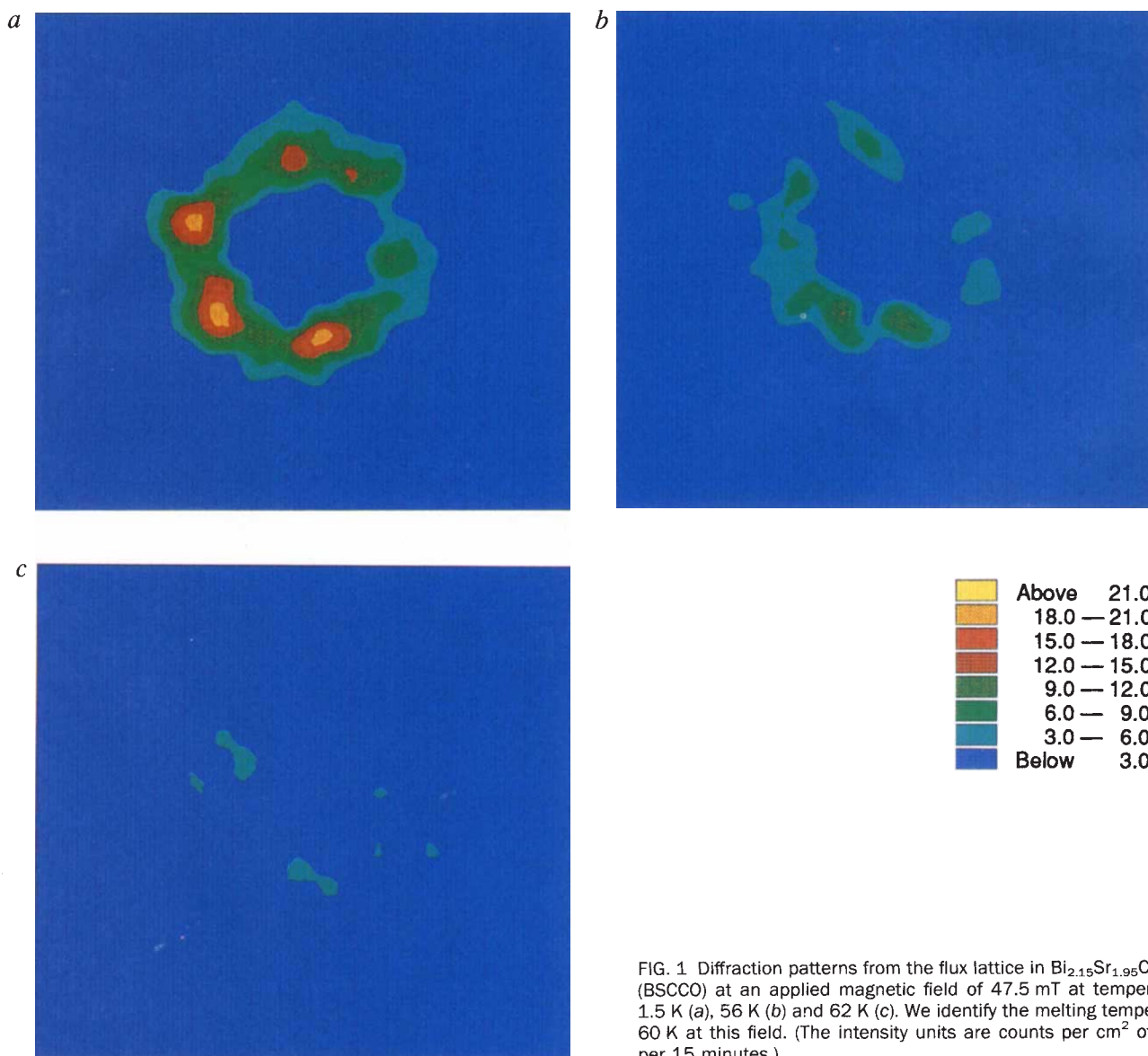


FIG. 1 Diffraction patterns from the flux lattice in $\text{Bi}_{2.15}\text{Sr}_{1.95}\text{CaCu}_2\text{O}_{8+x}$ (BSCCO) at an applied magnetic field of 47.5 mT at temperatures of 1.5 K (a), 56 K (b) and 62 K (c). We identify the melting temperature as 60 K at this field. (The intensity units are counts per cm^2 of detector per 15 minutes.)

theoretical prediction of flux line lattice melting given by the formula⁴

$$B_{3D}(T_m) \approx \frac{1}{[(4(\sqrt{2}-1)+1)^2 \pi \mu_0^2]} \times \left[\frac{\Phi_0^5 c_L^4}{(\gamma k_B T_c \lambda_{ab}(0))^2} \right] \left[\frac{1 - (T_m/T_c)^n}{T_m/T_c} \right]^2$$

where c_L is the Lindemann melting parameter, γ^2 is the effective mass anisotropy ratio, $\lambda_{ab}(0)$ is the zero-temperature value of the London penetration depth which arises from currents flowing in the a - b crystal planes, Φ_0 is the flux quantum and for a two-fluid model of superconductivity, $n=4$. The line is calculated using the values $c_L=0.2$, $\gamma=140$, $\lambda_{ab}(0)=1,800$ Å and $n=3.3$ (although there is some interplay between the values of these parameters, and some uncertainty about the prefactor in the equation). This line lies close to the melting transition independently observed in the currently unpublished μ SR measurements¹⁰. It is also very close to the irreversibility line, deduced from magnetic measurements on a small piece taken from the sample. It seems clear that at these fields the irreversibility line indeed represents the melting of the three-dimensional flux line lattice.

Within the accuracy of our data, we see no 'pre-melting' phenomena as the temperature is increased, such as a change in the width of the spots, only a fall in intensity. Above the melting transition, any diffracted intensity is too small for us to measure. There is no ring of intensity, as would be expected for a liquid array of rod-like flux lines. We believe that this is because the flux lines are not sufficiently straight in the liquid state. Using an expression for the tilt modulus c_{44} (ref. 15) (which should be independent of the presence of a lattice), and bearing in mind that the shear modulus c_{66} is zero in the liquid state, we may

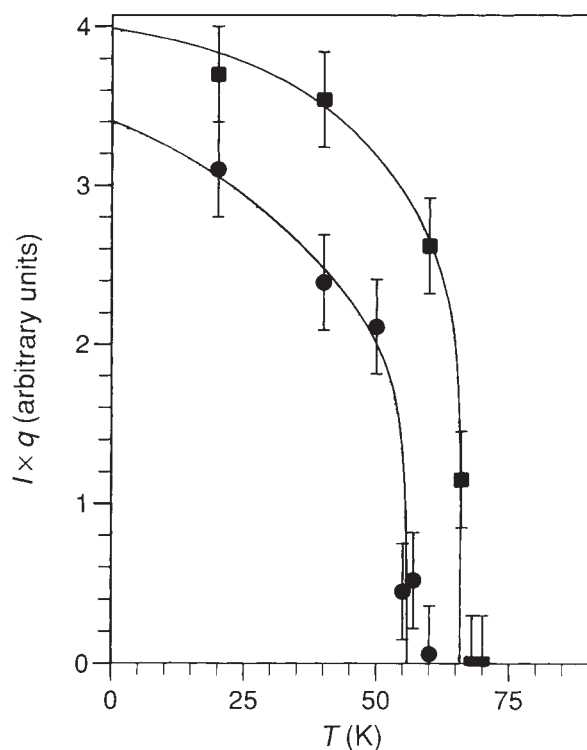


FIG. 2 Temperature dependence of the integrated intensity of one of the spots in the diffraction pattern, multiplied by q_{nk} (to give a quantity proportional to the form factor). Data is shown for 30 mT (squares) and 60 mT (circles). The solid lines are a guide to the eye.

estimate the energy to tilt a flux line by some fraction α of the flux lattice parameter d ($\sim \Phi_0/B$)^{1/2}, to form a kink of length L_z

$$E_{\text{tilt}} \approx \frac{2\Phi_0^2 \alpha^2 d^2}{\pi^2 \mu_0 \gamma^2 \lambda_{ab}^2 L_z}$$

Taking an extreme case of $\alpha=1$ and $L_z=d$, we find this energy is equivalent to a temperature of 13 K at $B=50$ mT. This model calculation leads us to expect that the flux lines will not be straight after the flux lattice has melted. Indeed, on increasing temperature and field the flux structure will eventually turn into a two-dimensional liquid 'pancake mix' (ref. 16), if it does not already form it on melting.

Decomposition of flux lines

We now turn to the variation of the diffracted signal at low temperature as a function of increasing field (as always, the new structure is formed by cooling through T_c in an applied field). We find that the signal disappears rapidly, with the midpoint of the transition at ~ 65 mT (Figs 4 and 5). The intensity of the spots is reduced in a similar manner to the temperature transition, although not quite so sharply. Related phenomena have been observed in the μ SR data¹⁰, and we ascribe this to a 'dimensional crossover' in pinning, which leads to a static arrangement of pancake vortices with little alignment in the field direction. According to the collective-pinning theory of Larkin and Ovchinnikov¹⁷, the length of a coherently pinned volume is $(c_{44}/c_{66})^{1/2}$ multiplied by its extent perpendicular to the field. In BSCCO, it is believed that flux lines are pinned individually¹⁸, so the perpendicular extent should be taken as d and the short-

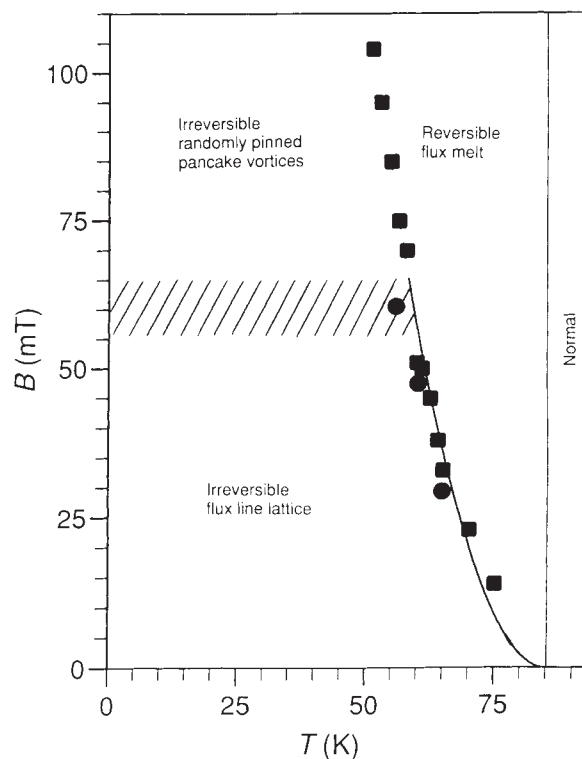


FIG. 3 B - T phase diagram showing the three-dimensional melting line, using $\gamma=140$, $\lambda_{ab}=1,800$ Å, $c_L=0.2$, $T_c=84$ K and $n=3.3$ (solid line). The crossover between flux lines and two-dimensional behaviour is represented by the hatched area, the circles are the melting transition (derived from the diffracted intensity) and the squares show the boundary between reversible and irreversible magnetic behaviour in hysteresis loops observed on a fraction of the same crystal (these measurements were made using a vibrating-sample magnetometer at a field sweep rate of 1 mT s⁻¹).

distance form of c_{44} should be used, which is extremely 'soft': using the expressions in refs 15 and 18, we find that

$$\frac{c_{44}}{c_{66}} \approx \frac{B^2}{\mu_0 \lambda_c^2 (\pi/d)^2} \cdot \frac{B \Phi_0}{16 \pi \mu_0 \lambda_{ab}^2} \approx \frac{1}{\gamma^2}$$

Simple substitutions show that the pinning length becomes comparable with the inter-CuO₂ plane separation s as the field is raised above $B_{2D} \approx \Phi_0/(s\gamma)^2$ (which is 47 mT with $s = 15$ Å and $\gamma = 140$); in view of the crudeness of our estimate of B_{2D} , this value is in sufficiently good agreement with the observed crossover field.

Further measurements below the melting line indicate that the crossover field B_{2D} is approximately temperature-independent. We represent it as the hatched area in Fig. 3. According to the recent μ SR measurements¹⁰, melting also occurs well above 65 mT; presumably this is two-dimensional melting, but the absence of a detectable diffracted signal indicates that the flux line structure is far too disordered in this region to be investigated by means of neutron diffraction.

At lower fields in the flux lattice state, the total intensity I_{hk} of a single diffracted spot, obtained by integrating over angle as the sample is rocked through the Bragg condition ('rocking curve') for the hk reflection, is given by the formula¹⁹

$$I_{hk} = 2\pi\varphi \cdot \left(\frac{\mu}{4}\right)^2 \frac{V\lambda_n^2}{\Phi_0^2 q_{hk}} |F_{hk}|^2$$

where φ is the incident neutron flux/m², μ is the magnetic moment of the neutron in nuclear magnetons, and F_{hk} is the form factor for the hk reflection, representing a Fourier coefficient of the field contrast in the mixed state. For rigid flux lines, the London theory gives

$$F_{hk} = \frac{B}{1 + (q_{hk}\lambda_{ab})^2}$$

where q_{hk} is the scattering vector.

A loss of intensity at a given sample orientation could arise either from a reduction in the form factor F_{hk} or an increase in the width of the rocking curve for the intensity. We have measured the low-temperature rocking curve both at 50 mT and

at 65 mT, where the intensity is a factor of 3.6 below that found at 50 mT. In both cases the FWHM of the rocking curve was 1.2 (2)[°] indicating that the form factor had changed. We believe that above B_{2D} the short length scale of the pinning in the c direction means that individual pancakes can be pinned away from the flux line axis, reducing the field contrast in the mixed state.

Now the rocking-curve width is closely related to the mosaic spread of the flux lattice, which is a measure of the straightness of the flux lines convolved with the effects of mosaic block length along the field (a 'mosaic block' is the size of a part of an imperfect crystal which may be considered as a small perfect crystal for the purposes of diffraction). The rocking-curve width implies an overall straightness of not worse than 1.2 (2)[°] combined with a block length of at least 10 μ m. It may seem hard to reconcile this with the short-distance wandering of the pancakes that we have postulated. However, we believe that it arises from the long-range forces that control c_{44} (the tilt modulus¹⁵, which make the lines 'stiff' over long distances but allow pancake displacements over short distances.

An alternative explanation of our results is that our sample is inhomogeneous, and that at 65 mT we are seeing the small fraction of the sample where the flux line lattice has not been destroyed by the field. However, the sharpness of the melting transition observed by μ SR¹⁰ does not support this explanation.

We may use the equations for the diffracted intensity and the form factor to calculate a value for λ_{ab} from spot intensities observed at 50 mT and 1.5 K. We obtain a value of 2,100 (200) Å which should be regarded as an upper limit, due to the effects of any disorder of the flux lattice on the diffracted intensity. This value is similar to that found by μ SR¹⁰ but is much shorter than the earlier accepted value of 3,000 Å (ref. 20). It should be noted that recent estimates from magnetization measurements, taking into account the effects of thermal fluctuations, give results rather closer to our value²¹.

Flux lattice versus vortex glass melting?

In one sense, a flux lattice should not exist in any real material, because it has been shown¹⁷ that arbitrarily weak pinning removes the long-range order of flux line arrangement. But if

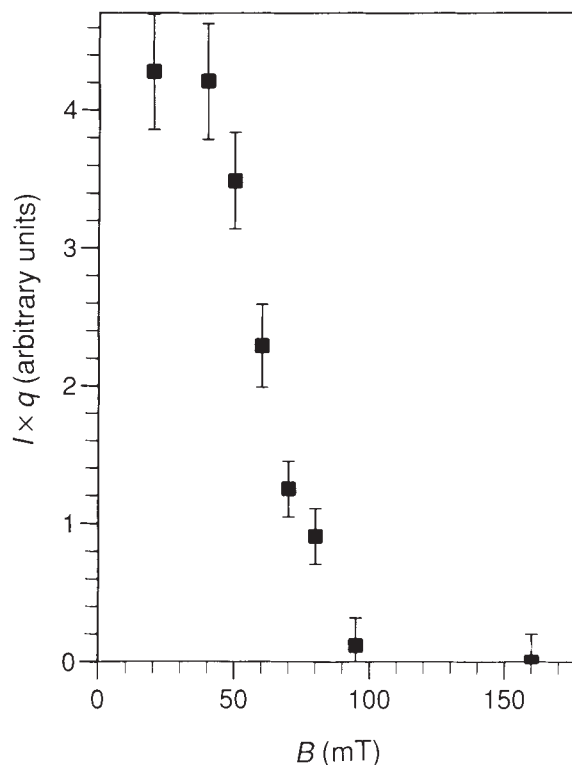


FIG. 4 Applied-field dependence of the integrated intensity (at 1.5 K) of one of the spots in the diffraction pattern multiplied by q_{hk} to give a quantity proportional to the form factor.

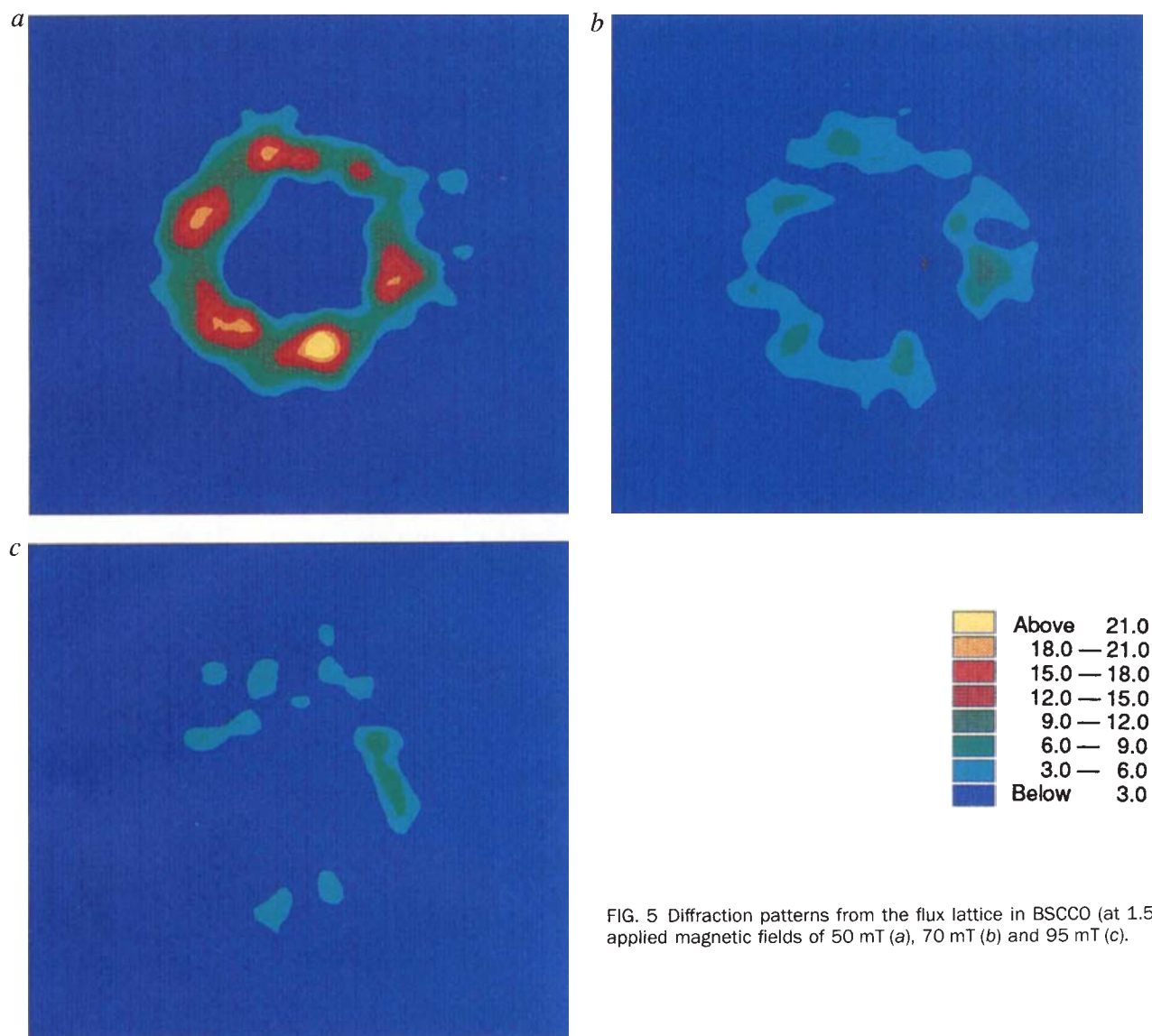


FIG. 5 Diffraction patterns from the flux lattice in BSCCO (at 1.5 K) at applied magnetic fields of 50 mT (a), 70 mT (b) and 95 mT (c).

the short-range order extends over a large enough distance, diffraction patterns will still be observed, particularly with a low-resolution arrangement like ours. Furthermore, the flux lattice may be expected to be an operationally useful concept if melting can be described by theories which ignore pinning⁴. This seems to be the case for our sample, and also for very 'clean' YBCO crystals, which show small hysteretic jumps in resistivity in a field; these jumps are expected to be the signs of a first-order melting transition²². However, disorder and pinning by twin planes remove these sharp transitions²³, and replace them by flux-glass-like melting¹. All the neutron-diffraction observations to date on flux line structures in YBCO report strong pinning

effects of twin planes (see, for example, refs 8, 9 and 12), and there have been no clear microscopic observations of flux lattice melting in these crystals. The absence of twin planes and the high anisotropy of BSCCO tend to push the melting transition well below T_c (where there are stronger signals) if the flux lines are ordered. This has allowed us to observe the fascinating phenomenon of flux lattice melting in this material. Because we find that flux lattice melting is associated with the appearance of electrical resistivity, our results emphasize the importance of low anisotropy and strong pinning (as well as high T_c), in ensuring the usefulness of these materials in magnetic fields at elevated temperatures. □

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Association between proto-oncoprotein Rel and TATA-binding protein mediates transcriptional activation by NF- κ B

Lawrence D. Kerr^{*}, Lynn J. Ransone^{*}, Penny Wamsley, Mark J. Schmitt, Thomas G. Boyer[†], Qiang Zhou[†], Arnold J. Berk[†] & Inder M. Verma

Molecular Biology and Virology Laboratory, The Salk Institute, PO Box 85800, San Diego, California 92138, USA

[†] Department of Microbiology and Molecular Genetics, Molecular Biology Institute, University of California at Los Angeles, Los Angeles, California 90024-1570, USA

The c-Rel protein is able to associate *in vitro* and *in vivo* with the TATA-binding protein (TBP) of the TFIID complex. Coexpression of TBP with c-Rel augments transactivation from the κ B site in *Drosophila* Schneider cells. DNA-binding mutants of TBP not only fail to cooperate, but they repress transactivation by c-Rel. There may be a direct communication between κ B enhancer binding proteins and basal transcription factors which leads to enhanced transcription.

The accurate initiation of transcription by RNA polymerase II is predicated by the dynamic interactions between two different classes of regulatory transcription factors. The first are a set of ubiquitous general factors required for the recognition of the transcriptional start site in the formation of the preinitiation complex and the subsequent recruitment of the polymerase. The second set of activator proteins recognize site-specific sequences in a promoter-specific way and are required to mediate an increase in the rate of transcription. *In vitro* transcription studies using crude extracts from HeLa cells supplemented with either tissue-specific^{1,2} or stimulated/inducible factors^{3,4} demonstrate that the basal factors on core promoter elements and the factors associated with enhancer elements can interact functionally. We have studied whether one class of enhancer binding protein, the Rel/NF- κ B family of transcription factors, induces κ B transactivation by direct interaction with the basal transcriptional machinery. The class of Rel homology domain transcription factors include v-Rel, encoded by the transforming gene of reticuloendotheliosis virus strain T and its cellular cognate c-Rel (ref. 5) and Xrel1 (ref. 6); the RelB protein⁷; the subunits of the purified NF- κ B complex, NFKB1 p110/p50 (refs 8, 9), RelA/p65 (refs 10, 11); the NFKB2 p105/p52 protein^{12,13}; and the *Drosophila* morphogen, Dorsal¹⁴. These all have a highly conserved region of ~300 amino acids in their amino termini (the Rel homology domain, RHD), which is responsible for DNA binding, homo- and heterodimerization, nuclear targeting and inhibitor (I κ B) binding. In addition, c-Rel, RelA/p65 and Dorsal each contain transcriptional transactivation domains at their carboxy termini¹⁵⁻¹⁷. All RHD proteins isolated so far are subject to cytoplasmic retention and inhibition of their DNA-binding properties by the I κ B family of inhibitor proteins. Once the inhibitory activity of the specific I κ B protein has been inactivated, the κ B complex translocates to the nucleus for association with its high-affinity κ B site.

Many basal transcription factors, including TFIIA, -B, -D, -E, -G/J, -H and -I, act in conjunction with RNA polymerase II in the initiation and regulation of transcription¹⁸. The formation of the preinitiation complex is coordinated by the binding of the TATA box-binding protein (TPB, or TFIID τ) and associated factors to the TATA element. Although TBP is only one compo-

nent of TFIID and cannot substitute for chromatographically purified TFIID in reconstituted transcription analysis^{19,20}, it can interact with all of the following: the TBP-associated factors (TAFs²¹⁻²³); the basal transcription factors TFIIA and TFIIB²⁴⁻²⁶; negative regulatory factors such as NC1, NC2, Dr1 (refs 27, 28); the RNA polymerase II large subunit²⁹; the viral transactivators VP16 (refs 30, 31), Epstein Barr virus Zta³² and adenovirus 13S E1A^{33,34}; and certain enhancer-specific factors (for example, ATF³⁵, SRF³⁶).

We show that TBP can associate specifically and with high affinity with c-Rel *in vitro* and *in vivo* in an interaction that involves the N-terminal residues of the Rel homology domain of c-Rel and the C-terminal basic domain of TBP. We suggest that transactivation by c-Rel requires a physical interaction with basal transcription factors in order to modulate an increase in κ B-enhancer-mediated transcription.

Association of κ B-binding factors with hTBP

Radiolabelled κ B site-binding proteins translated *in vitro* were tested for their ability to bind to bacterially produced human TBP. Figure 1a shows that of the members of the RHD superfamily, only c-Rel (lane 2), RelA/p65 (lane 4), and Dorsal (lane 7) can associate with TBP *in vitro*. No association is detected with v-Rel, p50, p52, or a control in which Sepharose-coupled glutathione-S-transferase (GST) is used in place of the GST-TBP fusion protein.

To ensure that c-Rel and TBP are interacting with one another, proteins produced and purified from *Escherichia coli* were used in a co-immunoprecipitation assay. Purified c-Rel protein from bacteria was phosphorylated *in vitro* by the catalytic subunit of cyclic AMP-dependent protein kinase in the presence of [γ -³²P]ATP, isolated by electrophoresis, and complexed in the absence or presence of unlabelled purified TBP. Immunoprecipitation with anti-TBP serum demonstrates that c-Rel co-immunoprecipitates only in the presence of TBP (Fig. 1b, lane 3). c-Rel is not co-immunoprecipitated with preimmune serum, nor when TBP is absent (lanes 1 and 2, respectively), indicating that no additional proteins are necessary for the interaction of c-Rel and TBP.

To delineate the region(s) of c-Rel that interact with TBP, deletion mutants of the murine c-Rel complementary DNA were transcribed and translated *in vitro* and assayed for their ability to bind to TBP. All proteins generated from the 3' end-deletion mutants were able to associate with TBP (summarized in Fig. 1c), suggesting that TBP interacts directly in the RHD. Only

^{*} Present addresses: Vanderbilt University School of Medicine, Department of Microbiology and Immunology, Nashville, Tennessee 37232, USA (L.D.K.); and Signal Pharmaceuticals Inc., 11545 Sorrento Valley Road, Suite 315, San Diego, California 92122, USA (L.J.R.).

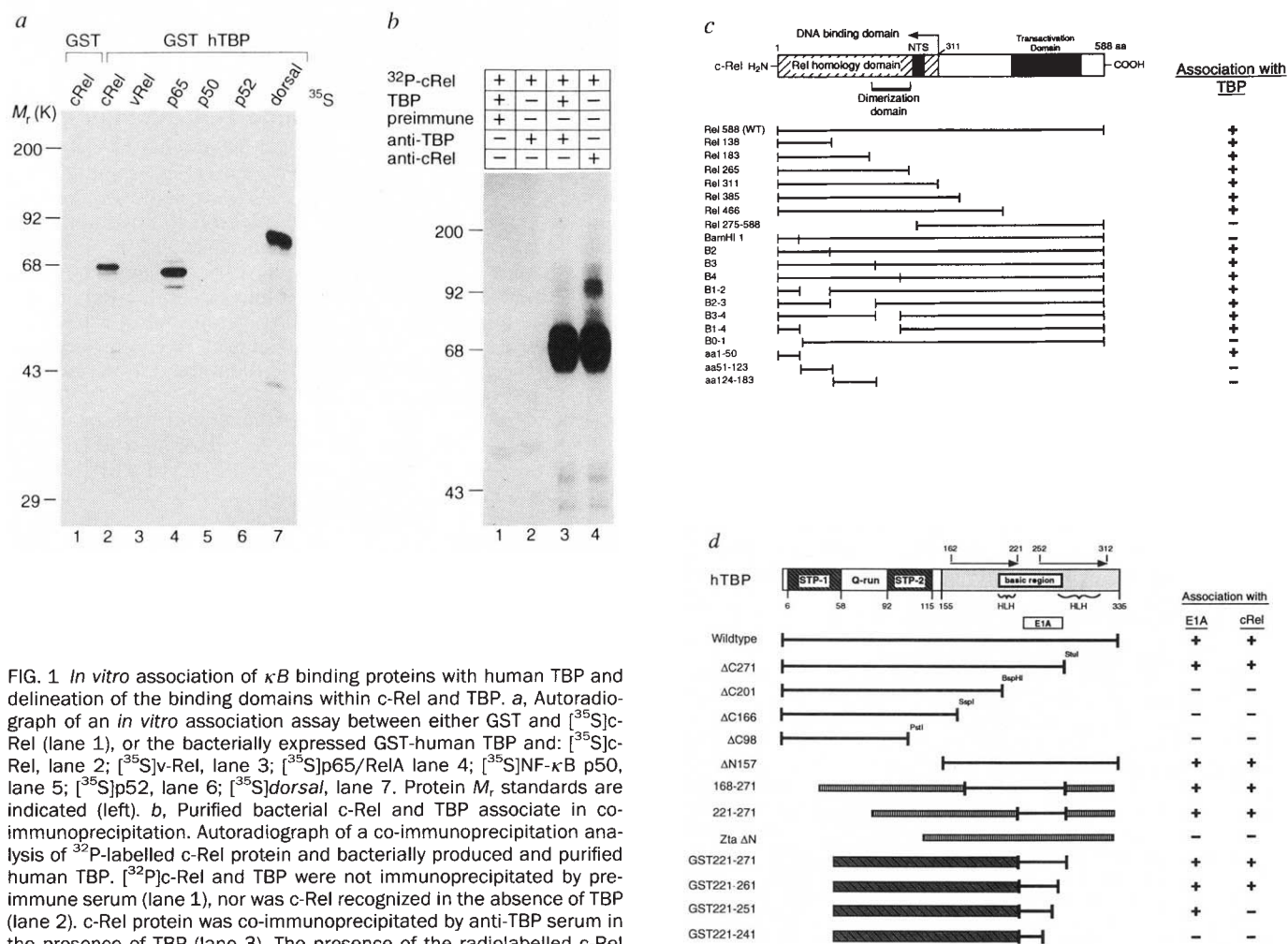


FIG. 1 *In vitro* association of κ B binding proteins with human TBP and delineation of the binding domains within c-Rel and TBP. **a**, Autoradiograph of an *in vitro* association assay between either GST and [³⁵S]c-Rel (lane 1), or the bacterially expressed GST-human TBP and: [³⁵S]c-Rel, lane 2; [³⁵S]v-Rel, lane 3; [³⁵S]p65/RelA lane 4; [³⁵S]NF- κ B p50, lane 5; [³⁵S]p52, lane 6; [³⁵S]dorsal, lane 7. Protein M_r standards are indicated (left). **b**, Purified bacterial c-Rel and TBP associate in co-immunoprecipitation. Autoradiograph of a co-immunoprecipitation analysis of ³²P-labelled c-Rel protein and bacterially produced and purified human TBP. [³²P]c-Rel and TBP were not immunoprecipitated by pre-immune serum (lane 1), nor was c-Rel recognized in the absence of TBP (lane 2). c-Rel protein was co-immunoprecipitated by anti-TBP serum in the presence of TBP (lane 3). The presence of the radiolabelled c-Rel protein was confirmed by co-immunoprecipitation with anti-c-Rel serum (5331; ref. 44) (lane 4). **c**, Insertion and deletion mutants of the murine c-Rel protein and the indications of either association (+) or no association (-) with GST-hTBP as assayed *in vitro*. aa, Amino-acid residue positions; WT, wild type. **d**, Schematic representation of TATA-binding protein cDNA mutants' ability to associate with either c-Rel or E1A protein. TBP deletion mutant proteins were associated *in vitro* with GST-c-Rel or GST-E1A. A plus sign denotes a significant association between the factors; a minus sign denotes no association detectable.

METHODS. **a**, The κ B binding factors were transcribed and translated *in vitro* (TNT, Promega) in the presence of ³⁵S-methionine/cysteine: c-Rel (pBSRel¹³⁵); v-Rel (pBRvRel¹⁴⁴); RelA/p65 (pBS65; ref. 10); p50 (pBS110; ref. 9); NFkB2/p52 (pBS49; ref. 12); dorsal (pBSdl⁴⁹). *In vitro* association was assayed as described³⁸, with minor modifications. The translation products (5 μ l out of 50 μ l reaction) were analysed by SDS-PAGE and processed for autoradiography. Equal amounts of radiolabelled protein were incubated in 400 μ l NETN (100 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0, 0.5% NP-40) with Sepharose-GST (2–5 μ g, 10–20 μ l) for 1 h at 25 °C, with rocking. The Sepharose-GST complexes were pelleted for 30 s at 14,000g and the supernatant was removed to a fresh tube. Proteins were incubated with Sepharose-GST-TBP (15 μ l, about 1–2 μ g) for 1 h at ambient temperature with rocking before extensive washing in NETN plus 0.005% SDS. Products were analysed by SDS-PAGE. The autoradiogram was exposed for 6 h at -70 °C. **b**, c-Rel protein purified from *E. coli* was phosphorylated *in vitro* by the catalytic subunit of cAMP-dependent protein kinase in the presence of [γ -³²P]ATP and subsequently purified by gel electrophoresis. Radiolabelled c-Rel (2 $\times$ 10⁵ c.p.m.) was combined with 40 ng purified bacterial human TBP (Promega) where indicated (+). Immune complexes were recognized by protein A-Sepharose and washed extensively in NETN buffer as already described. Precipitated products were analysed by SDS-PAGE. The autoradiogram was exposed for 2.5 h at -70 °C. **c**, pBSRel was digested with *PvuII* (expressing 1–138 amino acids), *PstI* (1–183), *StuI* (1–265), *DdeI* (1–311), *MscI* (1;385), *SalI* (1–466) or *EcoRI* (expressing amino acids 275–588). *BamHI* sites were inserted in frame by oligo-

nucleotide site-directed mutagenesis at positions 174 bp (B1), 393 bp (B2), 589 bp (B3) and 699 bp (B4). Construct B1–2 denoted that the *BamHI* fragment between B1 and B2 was removed. The mutant c-Rel cDNAs (1 μ g) were transcribed and translated *in vitro* according to the manufacturer's protocol (TNT, Promega) in the presence of Expre³⁵S (New England Nuclear-Dupont) using either T3 or T7 RNA polymerase, and the products (5 μ l) were analysed by SDS-PAGE. Equal quantities of protein were analysed by *in vitro* association assay with GST-TBP as in **a**. pTM-TFIIDAN157 (ref. 32; encodes the 178 amino acids of the conserved core domain of TBP) was transcribed and translated with ³⁵S-methionine. 7 μ l AN157 was assayed by association with either Sepharose-GST-c-Rel, Sepharose-GST-c-Rel(amino acids 1–50), Sepharose-GST-c-Rel (51–123), or Sepharose-GST-c-Rel(124–183). **d**, The TM-TFIID cDNA³² was digested with the enzymes shown to generate 3'-end-deleted constructs. The basic region of the conserved domain of TBP is sufficient to bind c-Rel. TBPAN157 encodes the 178 amino acids of the conserved core domain. The Zta transactivator of Epstein-Barr virus and the Zta cDNA bearing the nucleotide insert encoding the basic domain of hTBP (Zta/BP221-271; ref. 32) were translated *in vitro*, analysed by SDS-PAGE and used in association with GST-c-Rel. The 51-amino-acid basic region is sufficient to bind radiolabelled Rel. The GST-TBP nested 3'-deletion inserts were generated by polymerase chain reaction using the following oligonucleotides, which insert a *BamHI* site at the 5' end and a translation termination sequence preceding an *EcoRI* site at the 3' end: TFIIDPCR:Bam221, 5'-CGTGGATCC-TCTTGCACAGGAGCAAGAGTGAAGAA-3'; TFIIDPCR:Eco271, 5'-AAGTG-AATTCATTATTCTAACCTTATAGGAAACCTTAC-3'; TFIIDPCR:Eco261, 5'-AAGTGAATTCATTACCCACCATGTTCTGAATCTTGA-3'; TFIIDPCR:Eco-251, 5'-AAGTGAATTCATTAGAACTTAGCTGGAAACCAACTT-3'; TFIIDPCR:Eco241, 5'-AAGTGAATTCATTAACTCTAGCATATTTCTTGCTGC-3'. The E1A (135) cDNA (pTM-E1A135) has been described³³. The GST-E1A fusion construct was generated by inserting the *NcoI*/*StuI*-blunted insert (868 bp) into the *NcoI*/*HindIII*-blunted site of pGM-GST.

mutations that either interfered with (such as B1 in Fig. 1c) or removed (such as B0-1 in Fig. 1c) the first 50 amino acids of c-Rel affected the Rel-TBP interaction. To investigate the association of this region with TBP, GST-Rel fusion proteins were assayed that expressed the first 50 amino acids, or, as controls, amino acids 51–123 and amino acids 124–183. A construct expressing the first 50 amino acids supports the association with TBP to an extent comparable with the full-length c-Rel protein (Fig. 1c), suggesting that a region contained in the first fifty amino acids of the sequence is both necessary and sufficient for the association of c-Rel with TBP.

In a reciprocal experiment, we delineated the domain of TBP that interacts with c-Rel. We used C-terminal deletion mutants of human TBP to show that truncations removing sequences 3' to the *Bsp*HI site (amino acid 201) are unable to associate with GST-c-Rel (Fig. 1d). Removal of 157 amino acids from the N terminus (Δ N157), which contains the core domain that associates with E1A, gave a peptide able to bind c-Rel as effectively as wild-type TBP. A construct expressing only the basic region (amino acids 221–271) as an insert in the Epstein-Barr viral Zta cDNA³² can also associate with c-Rel as well as wild type. This basic region by itself is therefore sufficient to bind c-Rel. GST fusion constructs expressing amino acids 221–271 and amino acids 221–261 can both bind c-Rel. Deletion mutagenesis

revealed that the C-terminal boundary lies between residues 251 and 261 and that N-terminal boundary maps to at least amino acid 221.

c-Rel binds to TBP in the holo-TFIID complex

We have shown that the N-terminal 50 residues of c-Rel can bind to a 41-residue region of TBP *in vitro* (Fig. 1c and d). But in the cell, TBP is bound to other TAFs which are required for activated transcription *in vitro*^{21, 23}. We refer to this complex as holo-TFIID to distinguish it from the single TBP polypeptide. This complex contains at least nine TAFs²³, raising the question of whether the surface of TBP that interacts with c-Rel *in vitro* is exposed and available for interaction with c-Rel when TBP is assembled into the holo-TFIID complex. We addressed this question by assaying the ability of c-Rel to bind to the isolated holo-TFIID complex (Fig. 2).

Affinity matrices were prepared containing epitope-tagged TBP expressed in and purified from *E. coli*, or holo-TFIID prepared from HeLa cells expressing epitope-tagged TBP²³. These molecules were bound to monoclonal antibody 12CA5, which is specific for the epitope tag and which had been covalently linked to protein A-Sepharose. The matrices were incubated with ³⁵S-labelled c-Rel and then washed extensively. TBP, holo-TFIID and any bound c-Rel were then eluted from the matrices using

FIG. 2 c-Rel binds TBP in the holo-TFIID complex. **a**, Diagram of the experimental strategy. Affinity columns were prepared containing either epitope-tagged TBP (middle column) or epitope-tagged holo-TFIID complex (right column) bound to monoclonal antibody 12CA5 linked to protein A-Sepharose. 12CA5 protein A-Sepharose alone was used as a control (left column). **b**, Autoradiograph of SDS-PAGE of ³⁵S-radiolabelled c-Rel protein retained on 12CA5-reTBP beads (re, recombinant) (lane 4), 12CA5-holo-TFIID beads (lane 5), and, as a control, beads of 12CA5 linked to protein A-Sepharose without bound protein (lane 3) after elution with the appropriate peptide. Lane 1 shows *M_r* standards and lane 2, input ³⁵S-c-Rel. **c**, Western blot analysis confirms that TBP is present in the affinity matrices. Lane 1, bacterial epitope-tagged TBP(eTBP); lane 2, elution from 12CA5 column; lane 3, elution from 12CA5-reTBP column; lane 4, elution from 12CA5-holo-TFIID column. **METHODS.** **a**, Affinity resins containing human epitope-tagged TBP (12CA5-reTBP) or holo-TFIID (12CA5-holo-TFIID) were prepared as follows: for 12CA5-holo-TFIID, nuclear extract was prepared from HeLa cell line (LTRα3) expressing epitope-tagged human TBP and fractionated by phosphocellulose chromatography²³. The undialysed phosphocellulose 0.5–1.0 M KCl D fraction (1 ml of a ~2 mg ml⁻¹ solution) was incubated with 0.1 ml packed beads of anti-epitope monoclonal antibody 12CA5 covalently coupled to protein A-Sepharose with rocking at 4 °C for 8 h. The beads were then washed twice for 5 min each with 0.4 M KCl D buffer (20 mM HEPES, pH 7.9, 20% glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF) and twice for 5 min each with 0.1 M KCl D buffer. For preparation of 12CA5-reTBP, recombinant epitope-tagged human TBP was partially purified from engineered *E. coli* as described³³ and an amount roughly equivalent to the amount of reTBP present in 1 ml of the 0.1 M KCl D fraction from LTRα3 nuclear extract (measured by western blotting) was diluted into D buffer. This was then bound to 12CA5 linked to protein A-Sepharose and washed as described for the 12CA5-holo-TFIID column. 12CA5-reTBP beads, 12CA5-holo-TFIID beads, and, as a control, beads of 12CA5 linked to protein A-Sepharose without additional bound protein (0.1 ml packed beads) were then independently incubated at room temperature for 75 min in 0.5 ml 0.1 M KCl D buffer plus 0.045 ml rabbit reticulocyte lysate containing ³⁵S-labelled c-Rel protein translated *in vitro* as described. Beads were then washed 4 times for 5 min each in 0.1 M KCl D buffer, and subsequently eluted in 0.05 ml with the influenza epitope oligopeptide YPYDVPDYA at 1 mg ml⁻¹ in 0.1 M KCl D fraction buffer as described²³. Duplicate sets of sample aliquots from the resulting column eluates were then resolved by electrophoresis in 10% SDS-polyacrylamide gels. One set of samples was analysed by western blotting using mAb 12CA5 to ensure that roughly equivalent amounts of reTBP were present in the samples eluted from the 12CA5-reTBP beads and the 12CA5-holo-TFIID beads. The remaining sample set was subjected to 1 M sodium salicylate fluorography to measure the bound and eluted c-Rel. c-Rel bound to holo-TFIID complex at least as well as to the isolated TBP subunit.

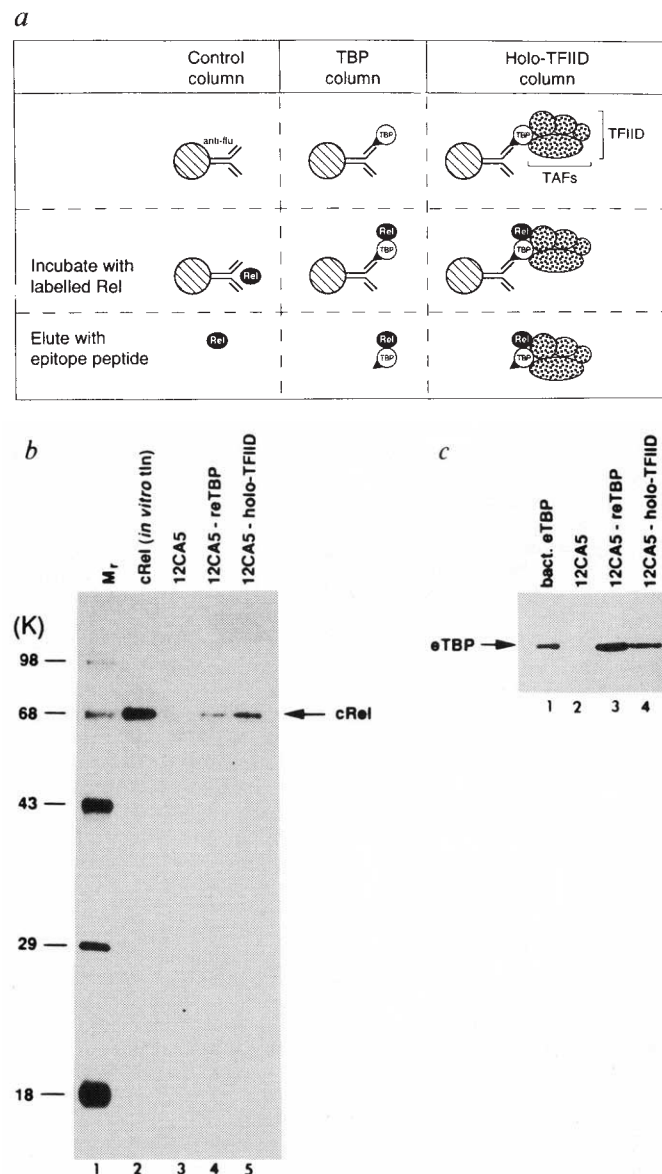
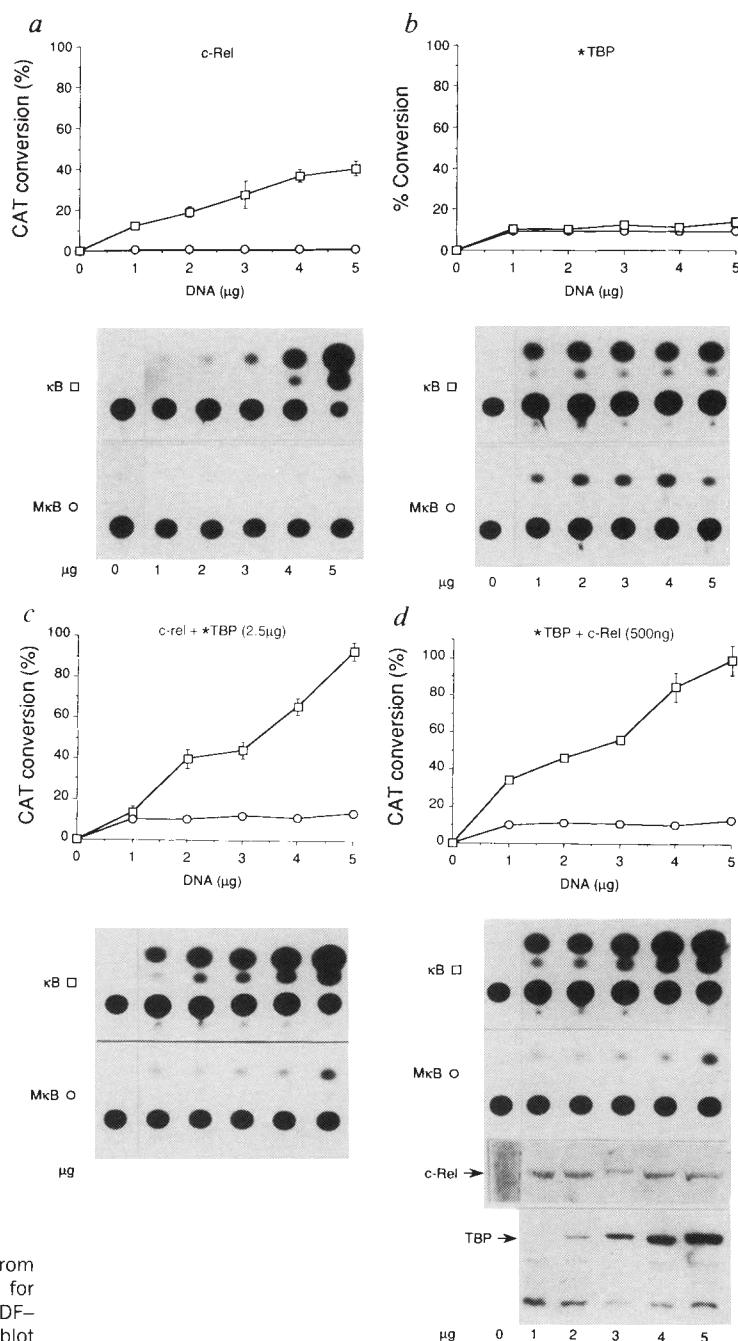


FIG. 3 Overexpression of TBP synergizes with c-Rel in inducing κ B-mediated transcription *in vivo*. **a**, The dose response of increasing A5'c-c-Rel on κ B transactivation. Schneider cells were transfected with either 6 $\times$ κ BAdhCAT or 6 $\times$ Mut κ BAdhCAT either alone or in the presence of increasing concentrations of A5'c-c-*rel*. Cell lysates were prepared, assayed for β -galactosidase activity, and equal protein amounts analysed for chloramphenicol acetyltransferase (CAT) activity. The percentage of [14 C]chloramphenicol converted to the acetylated forms was determined. **b**, Increasing the concentration of A5'c-TBP increases basal transcription regardless of the enhancer element. Schneider cells were transfected with either 6 $\times$ κ BAdhCAT or 6 $\times$ Mut κ BAdhCAT either alone or in the presence of increasing concentrations of A5'c-influenza epitope-tagged (asterisk) *Drosophila* TBP (1–5 μ g)³⁷. The percentage of [14 C]chloramphenicol converted to the acetylated forms was determined. **c**, c-Rel and *TBP synergize to increase κ B-mediated transcription. Schneider S2 cells were transfected with reporter genes as before. A constant amount of 'flu-tagged TBP (2.5 μ g) was transfected in the presence of an increasing amount of A5'c-c-*rel*. The percentage of [14 C]chloramphenicol converted to the acetylated forms was determined. **d**, TBP can augment a fixed amount of transfected c-*rel*. Schneider cells were transfected with either 6 $\times$ κ BAdhCAT or 6 $\times$ Mut κ BAdhCAT in the presence of 500 ng A5'c-c-*rel* and an increasing concentration of A5'c-*TBP. Cell lysates were prepared, assayed for β -galactosidase, and equal amounts of protein were analysed for CAT activity. The percentage of [14 C]chloramphenicol converted to acetylated forms was determined. **METHODS**. Schneider cells were transfected with either 6 $\times$ κ BAdhCAT (1.5 μ g) or 6 $\times$ Mut κ BAdhCAT (1.5 μ g), either alone or in the presence of increasing concentrations of A5'c-c-*rel* (1–5 μ g) as described. *Drosophila* Schneider S2 cells were plated at 3×10^6 cells per 60-mm Petri dish in complete medium one day before transfection. Calcium phosphate precipitates were prepared with 12 μ g supercoiled DNA in Hank's buffered saline (pH 7.14) and incubated on ice for 20 min before adding dropwise onto cells. Cells were incubated for 2 d at ambient temperature before collection. Cell lysates were prepared in 0.25 M Tris-HCl, pH 7.2, by freeze-thaw cycles, assayed for β -galactosidase activity, and equal amounts of protein assayed for CAT activity as described³⁸. The amount of acetylated [14 C]chloramphenicol was determined by cutting out the appropriate areas of thin-layer chromatography sheets for 14 C scintillation counting, and also by phosphor-imager analysis (Molecular Dynamics) of 14 C incorporated. From each cell lysate, 50 μ g protein was separated by SDS-PAGE (8% for detection of Rel, 12% for analysis of TBP) and transferred to PVDF-immobilon. Protein immune complexes were detected in western blot analysis. Anti-TBP sera was diluted 1:100 in blocking buffer and anti-Rel was diluted 1:250. Immune complexes were detected using 125 I-labelled protein A at 1 μ Ci ml⁻¹. Filters were then washed twice with blocking buffer and once with Tris-buffered saline, air-dried and exposed to X-OMat film (Eastman Kodak) at -70°C . A5'c- β gal (2 μ g) was included as an internal transfection control. The total DNA content of each transfected plate was brought to 12 μ g with the A5'c parental vector. Cell lysates were prepared two days post-transfection and processed for β -galactosidase activity. An equal amount of protein was analysed for CAT activity. The graphs summarize five transfection experiments, with standard error indicated; representative autoradiographs are shown underneath. In **b**, Schneider cells were transfected with either 6 $\times$ κ BAdhCAT (1.5 μ g) or 6 $\times$ Mut κ BAdhCAT (1.5 μ g), either alone or with increasing concentrations of A5'c-influenza epitope-tagged (asterisk) *Drosophila* TBP (1–5 μ g) and 2 μ g A5'c- β gal. All plates were transfected with 12 μ g total DNA. Cell lysates were assayed as before. The parental AdhCAT plasmid displayed increased basal activity in the presence of A5'c-*TBP (not shown). Results are the summary of 5 experiments. In **c**, Schneider S2 cells were transfected with reporter genes as before. A constant amount of 'flu-tagged TBP (2.5 μ g) was transfected in the presence of an increasing amount of A5'c-c-*rel* (1–5 μ g). Together



with 2 μ g A5'c- β gal, total DNA was brought to 12 μ g with A5'c(Δ p). The results plotted are the summary of 5 independent experiments, as represented by the CAT autoradiogram. **d**, Schneider cells were transfected with 1.5 μ g 6 $\times$ κ BAdhCAT or 6 $\times$ Mut κ BAdhCAT in the presence of 500 ng A5'c-c-*rel* and an increasing concentration of A5'c-*TBP. A5'c- β gal (2 μ g) was transfected as before. Cell lysates were assayed 2 d post-transfection for β -galactosidase and CAT. In some experiments, 50 μ g of the remaining cell extract was fractionated by SDS-PAGE and transferred to nitrocellulose. Blots were routinely probed with either anti-vRel-Mid (1:250) or 12CA5 (mouse monoclonal against influenza epitope) as indicated (c-Rel and TBP arrows, respectively). Immune complexes were detected with 125 I-labelled protein A. The anti-c-Rel blot shown here demonstrates that equal amounts of c-Rel are being expressed. Furthermore, using the anti-'flu epitope, we can distinguish transfected *TBP from untagged endogenous TBP to observe the dose-dependent increase in TBP expression. Autoradiograms were exposed for 28 and 16 h respectively.

a high concentration of epitope peptide. Slightly more c-Rel eluted from the holo-TFIID matrix compared with the TBP matrix (Fig. 2b), even though the same volume of eluate from the TBP matrix contained more TBP than the eluate from the holo-TFIID matrix (Fig. 2c). Much less c-Rel was bound and eluted from a control matrix containing the 12CA5 monoclonal antibody alone. These results demonstrate that c-Rel binds to holo-TFIID at least as well as it binds to isolated TBP. Consequently, the surface of TBP that interacts with c-Rel must be exposed in the holo-TFIID complex.

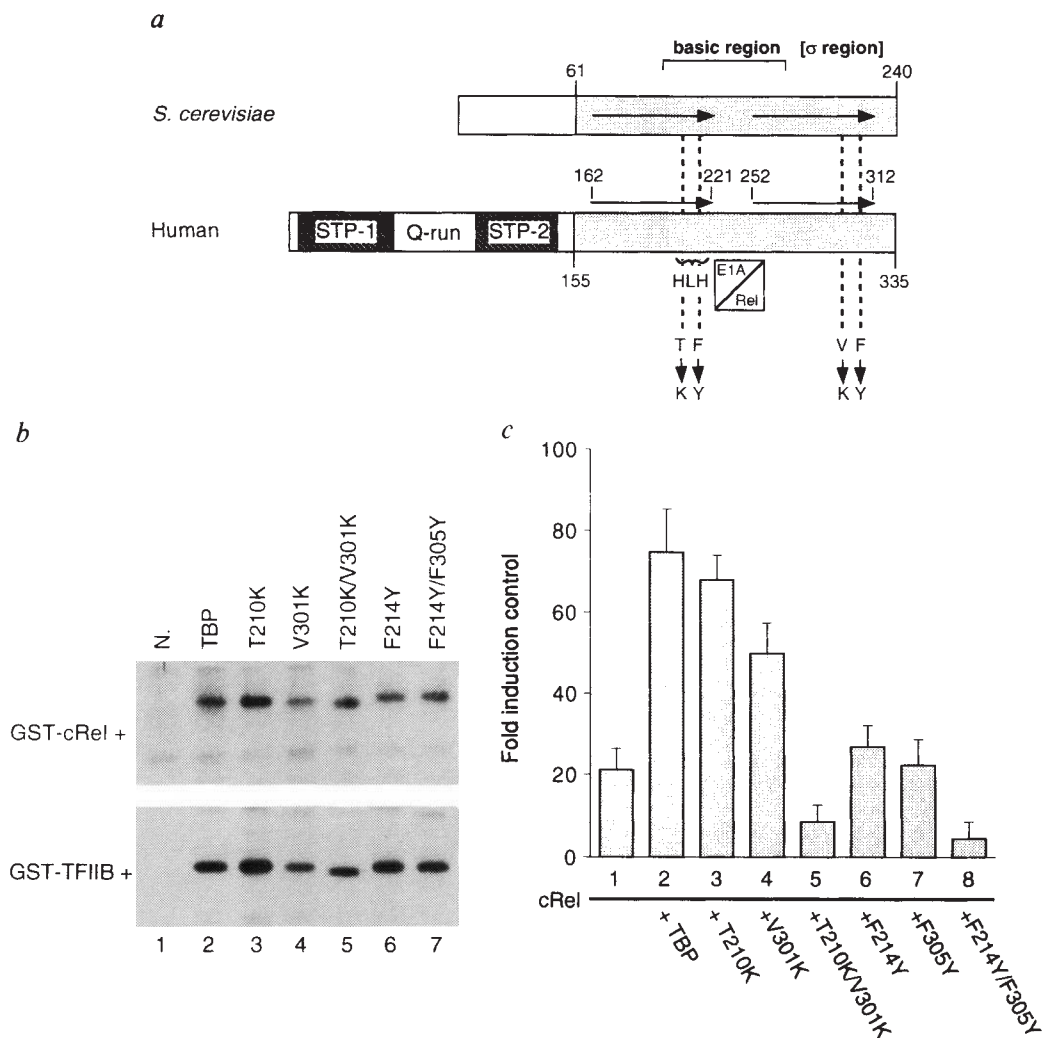
Cooperation of c-Rel and TBP *in vivo*

To determine whether the c-Rel/TBP interaction results in a biological response, we exploited the observation that TBP is

rate-limiting for TATA-box-containing RNA polymerase II promoters in *Drosophila* Schneider cells³⁷. We have previously used this cell system to follow specific κ B-mediated transactivation³⁸. Figure 3a shows that when Schneider cells are transfected with increasing amounts of actin 5'C-driven c-Rel plasmid, there is a linear increase in κ B-site dependent transactivation. Little or no transactivation is seen with the mutant κ B reporter construct. As already reported³⁷, an actin 5'C-driven influenza epitope-tagged *Drosophila* TATA-binding protein cDNA (*TBP) is able to increase the basal transcription of the minimal vector irrespective of the κ B site (Fig. 3b). To detect any increase in κ B-specific CAT activity, transfected levels of A5'C-*TBP were held constant while A5'C-c-Rel was increased. As shown in Fig. 3c, a strong cooperativity between *Drosophila* *TBP and c-Rel

FIG. 4 Dominant negative mutations of TBP block c-Rel transactivation *in vivo*.

a, Schematic representation of the direct repeat mutants of *S. cerevisiae* TBP and the corresponding residues in human TBP. The conserved core domain is shaded. The serine-threonine-proline (STP) regions and the glutamic acid stretch (Q-run) in human TBP are shown. The mutations occur in the helix-loop-helix (HLH) domain of the direct repeats. The arrow pointing downwards indicates that the upper amino acid has been changed to the lower amino acid, as described. b, Association of TBP mutants with GST-c-Rel, GST-TFIIB, and TATA DNA. ³⁵S-methionine-labelled TBP and TBP mutants translated *in vitro* were assayed by association with GST-c-Rel or GST-TFIIB as indicated. c, Effect of mutant TBP expression on c-Rel-mediated transactivation. *Drosophila* S2 cells were transfected with the reporter construct 6 × κ BAdhCAT in the presence or absence of the c-Rel expression vector, A5'C-c-Rel; each TBP expression vector was transfected, as well as 2 μ g A5'C- β gal. Cell



lysates were processed for β -galactosidase and CAT activities. Results are the summary of 3 individual transfection experiments and are expressed as fold induction over control (per cent acetylation of c-Rel transfectants on κ B sites versus mutant κ B sites divided by the per cent acetylation of the κ B reporter construct alone). METHODS. a, Actflu TFIID was a gift from J. Colgan and J. Manley³⁷; yeast TBP clones were from S. Hahn⁵⁰. The dominant negative mutation of human TBP were generated by oligonucleotide site-directed mutagenesis of single-stranded pTM-hTFIID. All mutations were verified by sequence analysis. Two independent clones of each mutation were analysed in transfection experiments. The following oligonucleotides were used to generate the hTBP mutations: TFIID/DR1:T210K, 5'-ACTGAAATCAGTGCCTTTGGTTCGTGGCTCTCT-3'; TFIID/DR1:F214Y, 5'-CATTTTCCAGAACTATAATCAGTGCCTGGT-3'; TFIID/DR1:F214L, 5'-CATTTTCCAGAACTGAGAATCAGTGCCTGGT-3'; TFIID/DR2:V301K, 5'-AACAAAATAAGGAGTTAAATCTGGGTTTGAT-3'; TFIID/DR2:F305Y, 5'-

AACATTTTCCAGAAACGTAAATAAGGAGAACAAT-3'; TFIID/DR1:F197L, F214L, 5'-CCCAGAACTGAGAATCAGTGCCTGGTTCGTGGCTCTCTATCC-TCATGATACCGACGAGCCGCTTGGG-3'. The (NcoI/BamHI)/blunt insert of the TBP mutants were inserted in A5'C (Δ) BamHI/blunt. b, TFIIB cDNA was a gift from D. Reinberg. GST-TFIIB was generated by inserting a BamHI site in-frame with the initiating ATG by site-directed oligonucleotide mutagenesis. The resulting BamHI/EcoRI insert was cloned into GM-GST and assayed for production of the fusion protein. Products were analysed by SDS-PAGE and autoradiograms exposed for 10 h at -70 °C. c, The effect of mutant TBP expression on c-Rel-mediated transactivation. *Drosophila* S2 cells were transfected with the reporter construct 6 × κ BAdhCAT (1.6 μ g) in the presence or absence of the c-Rel expression vector, A5'C-c-Rel (2.5 μ g). Five μ g of each TBP expression vector was transfected as well as 2 μ g A5'C- β gal. Cell lysates were prepared and processed 2 d post-transfection for assay of β -galactosidase and CAT.

results in almost maximal chloramphenicol acetyltransferase (CAT) activity. These results are consistent with those in Fig. 3d, in which *TBP was increased while the amount of transfected c-Rel was held constant. Our attempts to use c-Rel mutants unable to bind TBP in transfection assays were complicated by the fact that mutations in the TBP binding domain also prevent c-Rel from binding to the κ B site (12 different point mutations; L.D.K., unpublished results). Nevertheless, our results support the *in vitro* association data and indicate that c-Rel and TBP

(as part of the TFIID complex) probably interact *in situ* and on a DNA template. Furthermore, we propose that c-Rel-mediated transactivation is in part due to interactions with the TBF of the TFIID complex.

Mutations block c-Rel-mediated transactivation

A bipartite DNA-binding domain has been defined for the conserved core domain of TBP through the use of point mutations in both the direct repeat regions that abolish DNA binding³⁹. Alterations in the direct repeats do not seem to alter interactions with TFIIA or E1A, which associate with the basic region⁴⁰ (L.D.K., unpublished observations). We have generated point mutations in the human TBP cDNA corresponding to those residues in *Saccharomyces cerevisiae* encoding the helix-loop-helix domain⁴¹. All TBP mutant proteins were able to associate with both GST-c-Rel and GST-TFIIB, another basal transcription factor that binds outside the basic region⁴² (Fig. 4b; lanes 1–7, top and bottom respectively). But only certain mutations (wild-type, T210K) effectively bind TATA-containing sequences (data not shown). Using the Schneider cell system to study cooperation in transactivation between c-Rel and the TBP mutants, we found that the single point mutant in the first direct repeat at threonine 210 (T210K) and at valine 301 (V301K) in the second repeat significantly increased transactivation of the multimerized κ B-CAT construct by c-Rel (Fig. 4c, lanes 3 and 4). TBP cDNA mutants in both direct repeats that can no longer bind DNA³⁹ (data not shown) are incapable of cooperating with c-Rel, but instead inhibit the extent of transactivation (Fig. 4c, lane 5). These results are consistent with the inability of the double point mutants of TBP to complement TBP-deficient yeast in viability assays⁴³. Other point mutations in the direct repeats, in which conserved phenylalanine residues are replaced (F214Y; F305Y), could not cooperate with c-Rel (lanes 6 and 7). Furthermore, a double mutation in F214Y and F305Y also blocks transactivation of the κ B promoter by c-Rel (lane 8). These observations suggest that TBP capable of binding a TATA site is required for synergistic transactivation by c-Rel. Furthermore, the dominant negative mutants of TBP decrease transactivation by c-Rel, probably as a result of interference with the endogenous TATA binding protein. Alternatively, mutated TBP may still bind to other essential basal factors necessary for efficient transcription and consequently influence it.

Co-immunoprecipitation of TBP and c-Rel

We next studied whether c-Rel and TBP could also interact *in vivo*. Upon stimulation with phorbol myristyl acetate (PMA) and phytohaemagglutinin (PHA), c-Rel (and the NF- κ B complex) is translocated to the nucleus, where κ B binding is detected within minutes. We prepared nuclear and cytoplasmic extracts from unstimulated and stimulated HeLa cells and tested for an association between cellular TBP and c-Rel. There is a specific association of nuclear human Rel (p86) with TBP in stimulated cells (Fig. 5, lane 6), whereas only a little human Rel is detected in unstimulated nuclei (Fig. 5, lane 2). As might be expected, TBP is detectable only in nuclear extracts from these cells, and exceeds the amount of c-Rel protein. Based on the results in Fig. 5, we estimate that ~10% of total cellular c-Rel protein translocates to the nucleus upon treatment, and that nearly all of this associates with TBP. About 5% of the total TBP in the cell associates with c-Rel.

c-Rel also associates with TFIIB

Because of the association of c-Rel, p65/Rel A and Dorsal with TBP, we investigated whether other components of the preinitiation complex interact with members of the κ B family. The association of TBP with TFIIB to effect the recruitment of RNA polymerase II suggested that TFIIB might be a candidate for an associated factor. Figure 6a shows that c-Rel interacts with *in vitro*-translated TFIIB *in vitro*. Furthermore, the domain(s) through which c-Rel and TFIIB associate is distinct from that

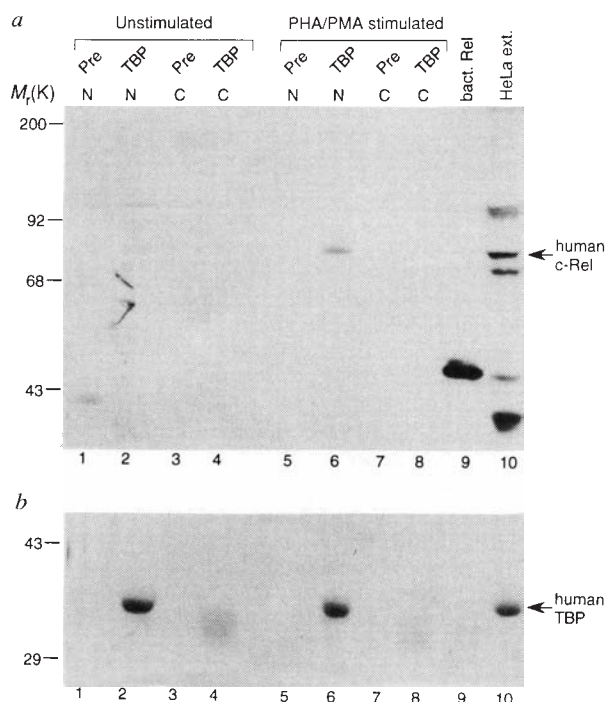


FIG. 5 Co-immunoprecipitation of TBP and c-Rel from activated HeLa cell nuclei. *a*, Anti-c-Rel serum recognizes a protein in anti-TBP treated lysates. Cytoplasmic (C) or nuclear (N) extracts from either stimulated (PMA + PHA) or unstimulated HeLa cells were first immunoprecipitated using anti-TBP serum before fractionation of immune complexes by SDS-PAGE and transfer to PVDF membrane. Immune complexes recognized by an anti-c-Rel serum are shown. Bact. Rel, truncated bacterial c-Rel, used as a positive control for the anti-c-Rel blot; HeLa ext., HeLa extract input before immunoprecipitation. *b*, The filter used in *a* was probed with anti-TBP serum to detect the presence of TBP in the immunoprecipitates.

METHODS. *a*, HeLa (S2) cells were either stimulated with PMA (Sigma; 100 ng ml⁻¹) and PHA (Sigma; 5 ng ml⁻¹) for 4 h at 37 °C, or left untreated before isolation of cytoplasmic (C) and nuclear (N) extracts. Protein concentration was determined by Bradford analysis (BioRad). 150 µg of each protein extract was incubated with either preimmune or anti-TBP in NETN for 6 h before addition of protein A-Sepharose. The precipitable immune complexes were washed four times in NETN. Bound proteins were separated from immunoglobulins by boiling in the presence of 5 mM iodoacetamide. Proteins were separated by SDS-polyacrylamide (10%) gel electrophoresis and transferred to PVDF immobilization. Nonspecific protein interactions were blocked in TBS (20 mM Tris-HCl, pH 7.5, 100 mM NaCl) containing 5% dried milk for 4 h at 4 °C with shaking. A 1:250 dilution of anti-v-Rel-Mid (gift from H. Bos), which recognizes the conserved Rel homology domain, was incubated in TBS plus 5% milk overnight at 4 °C. The blot was rinsed 4 times in TBS and 0.5% Triton X-100 before staining for immune-specific complexes with Proto-blot (Promega); total protein was stained with Coomassie blue. Bacterial truncated c-Rel (Rel 466) was used as a positive control (lane 9). HeLa nuclear extract (N) from stimulated cells was fractionated as a positive control for human c-Rel reactivity. *b*, After treatment with mild detergent, the filter was exposed to the anti-TBP serum used in the original immunoprecipitation. Immune complexes were visualized as a result of their reactivity with alkaline phosphatase (Protoblot, Promega), used to confirm that TBP had been immunoprecipitated from the extracts.

required for c-Rel with TBP, as the deletion mutant B0-1 (representing c-Rel residues 52–466), which does not bind TBP (Fig. 1c), does bind TFIIB (Fig. 6a). Although NF- κ B p50 fails to associate with TBP under our *in vitro* conditions (Figs 1 and 6b), it binds strongly with TFIIB (Fig. 6b) and requires a region outside direct repeat 1 (amino acids 122–198). We are at present mapping the exact domain(s) needed for association, and our results so far suggest that heterodimerization between κ B family members and RNA polymerase II factors might be critical in regulating the initiation of transcription.

Discussion

Dynamic interactions between proteins allow the cell to receive, communicate and interpret the myriad of extracellular signals. One profound effect of cell stimulation is the initiation of transcription of selected genes, whose products can result in cellular proliferation, differentiation, development, or death. What is the mechanism(s) by which genes can undergo an increase in the rate of basal transcription in response to a given stimulus? One clue to the answer comes from the identification of tissue- and stimulus-specific regulatory elements in the promoters of targeted genes, and another must lie in the interaction and modulation of factors that regulate the basal transcription of all eukaryotic class II genes.

We have shown that human TBP associates with specific members of the κ B binding family, namely, c-Rel, RelA/p65, and Dorsal (Fig. 1). These associations occur under stringent wash conditions, in the presence or absence of DNA, and require specific regions within each protein. Deletion mutagenesis of c-Rel, a prototypic member of the family of κ B-site-binding proteins, revealed that it was not the C-terminal transactivation domain but rather the N-terminal 50 residues that are necessary and sufficient for interaction with TBP (Fig. 1).

As the N-terminal region of c-Rel is adequate for association with TBP, we were surprised that v-Rel did not associate with TBP (Fig. 1a). Closer examination of c-Rel and v-Rel proteins revealed that during the biogenesis of v-Rel, the transforming gene of reticuloendotheliosis virus, the first nine amino acids of turkey c-Rel are deleted and replaced by viral Env protein. Unlike c-Rel, v-Rel represses transcription from a κ B site, pre-

sumably by making non-productive DNA-protein complexes⁴⁴. The dominant negative phenotype displayed by v-Rel can perhaps be explained not only by the lack of transactivation domain, but also by its inability to bind TBP and failure to form a preinitiation complex. The N-terminal sequences of c-Rel have also been implicated in redox regulation of Rel DNA binding activity through an RxxRxRxxC motif⁴⁵; we analysed point mutants of this motif and found that the association with TBP was unaffected. Point mutations in either arginine 29 or glutamic acid 37 dramatically reduce the association of c-Rel with TBP *in vitro*. But, as with most mutations the Rel homology domain, DNA binding was prevented (data not shown). Thus, association of c-Rel with TBP is independent of DNA-binding activity.

The basic region between the conserved direct repeats is critical for interaction with VP16 (refs 30, 31), with yeast acidic activator proteins⁴⁶, and with the adenovirus-2 13S E1A protein^{33,34}, and p53 (ref. 47) and TFIIA^{24,40} proteins. Deletion mutagenesis confirmed that this region is necessary for the 50 amino-terminal residues of c-Rel to interact with TBP (Fig. 1d). The crystal structure of one of the *Arabidopsis* TBPs has revealed that this region encodes helix H2, which lies on the outer surface of the 'molecular saddle' in a region readily accessible to other proteins⁴⁸. The fact that E1A and c-Rel compete for almost the same binding sites on TBP (data not shown) raises interesting points. Perhaps one mechanism by which viral transactivators usurp the normal cellular transcriptional machinery is to compete for basal transcription factors. E1A protein (from 13S mRNA) not only prevents the binding of c-Rel to TBP but can also displace it from a TBP template (L.D.K., unpublished observation). So if only one factor can occupy this site at a given time, then the particular function of TBP in the TFIID complex might be influenced by the associated factor. We have also observed direct physical association of basal transcription factor TFIIB with c-Rel (Fig. 6). But neither the region of c-Rel that interacts with TBP, nor the region of TBP (221–261) that associates with c-Rel binds TFIIB (Fig. 6; and L.D.K. and L.J.R., unpublished results).

We have shown that c-Rel and TBP co-immunoprecipitate in nuclear extracts from HeLa cells (Fig. 5). For most lymphoid cells,

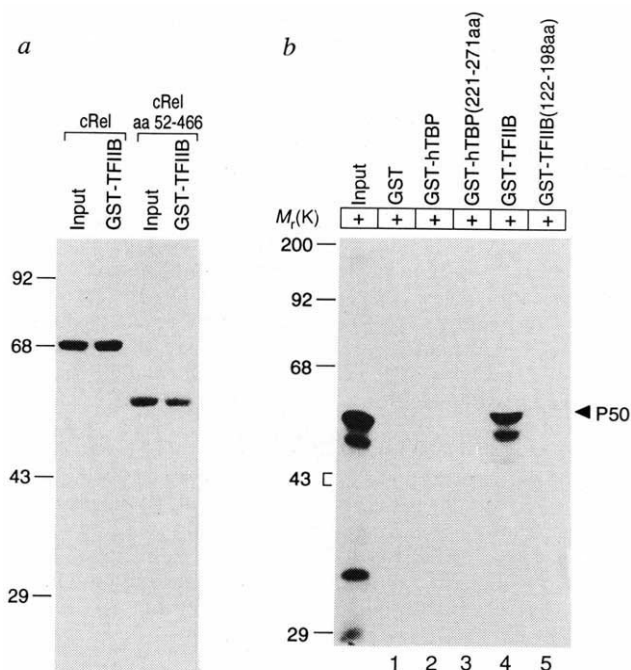


FIG. 6 Association of c-Rel and p50 with TFIIB. *a*, c-Rel interacts with TFIIB *in vitro*. Both *in vitro*-translated c-Rel and a deletion mutant of c-Rel, in which the first 50 amino acids (aa) and the transactivation domain (c-Rel amino acids 52–466) were deleted, bind the bacterial fusion protein GST-TFIIB in the *in vitro* association assay described in Fig. 1 legend. The autoradiogram has been exposed overnight. *b*, NF- κ B p50 fails to bind TBP but associates with TFIIB. Human NF- κ B p50 was translated *in vitro* and the resulting radiolabelled products assayed for their ability to associate with GST (lane 1); GST-hTBP (lane 2); GST-hTBP(221–271), which expresses only the basic region of TBP (lane 3); GST-TFIIB (lane 4); or GST-TFIIB(122–198), which expresses only the direct-repeat-1 domain of human TFIIB (lane 5); only full-length TFIIB was able to associate quantitatively. The autoradiogram was exposed for 12 h.

c-Rel is thought to represent <0.003% of total protein and is mostly sequestered in the cytoplasm, presumably in association with I κ B proteins until some (<10%) is translocated to the nucleus in response to external signals. Although we cannot measure the stoichiometry of the c-Rel and TBP association, we believe that the Rel protein that translocates to the nucleus binds only ~5–10% of the TBP available. This would not be surprising, given that for any specific stimulus numerous transcription factors are induced, many of which may interact with TBP. To assess the role of c-Rel and TBP association in the transcription of κ B-site-containing genes, we used the *Drosophila* Schneider cell system as an *in vivo* model for TFIID activation of TATA-containing promoters³⁷. A dose-dependent increase in c-Rel-mediated κ B enhancer-specific transactivation was observed (Fig. 3a), but overexpression of *Drosophila* influenza-epitope tagged TBP increased basal expression of the minimal alcohol dehydrogenase (Adh)-CAT reporter, regardless of whether the κ B or mutant κ B site was attached (Fig. 3b). Transactivating potential was synergistic, however, when both expression plasmids were transfected (Fig. 3c and d). Similarly, in co-transfection experiments, there is a strong synergy between Dorsal and TBP on a Dorsal-binding site/TATA reporter construct (J. Colgan, J. Norris, and J. Manley, personal communication). We have excluded the possibility that overexpression of TBP is increasing endogenous Dorsal levels as the Dorsal morphogen neither binds to nor transactivates the HIV-LTR κ B motif multimerized in our 6 $\times$ κ BAdhCAT construct. We found no significant alteration of either the actin 5'C-driven β -galactosidase or the actin 5'C-driven c-Rel expression construct in the presence of *TBP. We conclude that c-Rel and TBP can cooperate *in vivo* to activate κ B-containing promoters. Furthermore, double point

mutants that abolish the DNA-binding potential of TBP also block its ability to cooperate with c-Rel *in vivo* (Fig. 4c) while still allowing either c-Rel or human TFIIB to associate (Fig. 4b). We originally attempted to use the yeast dominant negative direct repeat mutants for such experiments and were surprised that it did not associate with either c-Rel or human TFIIB in our assays. It did not increase basal transcription of the Adh-CAT construct in the Schneider cell system (data not shown). These results are consistent with *in vitro* observations that human or *Drosophila* TFIID can function with Sp1 activator whereas yeast TFIID cannot. Our data do not exclude the possibility that activator proteins like c-Rel/NF- κ B function by associating with TBP to prevent its association with a negative transcription factor or to help in recruitment of other basal transcription factors such as TFIIA and TFIIB.

The association of c-Rel and p50 with TFIIB (Fig. 6) suggests that these activator proteins can interact with several members of the basal transcription machinery. It is intriguing that p50, which does not associate with TBP (Figs 1 and 6), can associate with TFIIB. In one model, p50/p65 or p50/c-Rel heterodimers, after binding to their cognate κ B-site, could associate with TBP via Rel or p65 and facilitate formation of the initiation complex by association with TFIIB through p50 and/or Rel/p65. Preliminary data indicate that the binding domain of c-Rel for TFIIB is different from that for TBP. The c-Rel protein favours several protein-protein interactions, including dimerization, binding of I κ B proteins, DNA binding, and association with TBP and TFIIB, and perhaps with other components of the transcriptional machinery. It will be important to establish whether c-Rel and other NF- κ B proteins can also have interactions directly with TAFs. □

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Evidence for an offset active nucleus in the Seyfert galaxy NGC3227

E. Mediavilla & S. Arribas

Instituto de Astrofísica de Canarias, 38200-Laguna, Tenerife, Spain

THE active nuclei of Seyfert 1 galaxies are characterized by a small (~ 0.03 pc) region of intense broad emission lines, indicative of gas moving at extremely high velocities and ionized by a non-thermal radiation field. These spectral features are usually explained by invoking the presence of a central supermassive black hole. Here we present two-dimensional spectra of the Seyfert galaxy NGC3227, from which we have calculated the intensity maps of the emission lines, and the velocity field representing the motion of gas within the central kiloparsec of the galaxy. Surprisingly, we find that the region of broad emission lines is offset from the kinematic centre by about 250 pc, suggesting that the putative supermassive black hole is displaced from the true galactic centre. Possible explanations of this finding include the transient migration of the black hole from the galactic centre, a black hole in orbit about a central concentration of dark matter, or the capture of the black hole during the merger of NGC3227 with another galaxy.

NGC3227 is a spiral galaxy with two well-defined arms, one of which ends in the elliptical companion galaxy NGC3226 (ref. 1). De Vaucouleurs *et al.*² have suggested that NGC3227 is partially barred (SABa pec in their classification scheme), but there is no general agreement in this respect³. This galaxy belongs to the group of Seyfert 1 galaxies, which are distinguished from Seyfert 2's by the presence of very strong and broad H and He permitted emission lines (broad emission lines, or BELs) in the spectra of their nuclei. The broad lines correspond to gas velocities of $\sim 10^3$ to 5×10^3 km s⁻¹. Their short-term variability⁴ (months) of these lines suggests that they originate in a small region, approximately a light month (0.03 pc) across. Both Seyfert 1 and Seyfert 2 galaxies exhibit narrower permitted and forbidden emission lines (narrow emission lines, or NELs) with widths corresponding to velocities of the order of a few hundred km s⁻¹. These lines come from a much more extended region, and can be spatially resolved for some Seyfert galaxies. In a low-activity state, the spectrum of NGC3227 resembles that of a Seyfert 2 galaxy because the BELs can vanish, this being the reason why NGC3227 was initially misclassified as a Seyfert 2 (ref. 5).

In the standard model, the nuclei of Seyfert galaxies are thought to be powered by the accretion of matter onto a supermassive black hole. For a Seyfert 1 this should reside in the broad-line region (BLR, the region giving rise to the broad lines) which is thought to be situated at the kinematic centre of the

TABLE 1 Optical nucleus and systemic velocities

v_{opt} (km s ⁻¹)	Source	v_{sys} (km s ⁻¹)	Source
$1,085 \pm 15$	NEL, 1989 data	$1,136 \pm 15$	NEL, 1989 data
$1,070 \pm 5$	[O III] ¹⁶ , C80	$1,145 \pm 28$	Absorption lines ¹⁶
$1,102 \pm 15$	NEL, 1991 data	1,140	Absorption lines ¹⁷
$1,063 \pm 25$	[O III] ¹⁸ , (C50 + v_{pk})/2	1,156	CO ¹⁹
—	—	1,146	H I ²⁰
—	—	$1,138 \pm 15$	H I ²¹
—	—	(1,106)	H I ²²

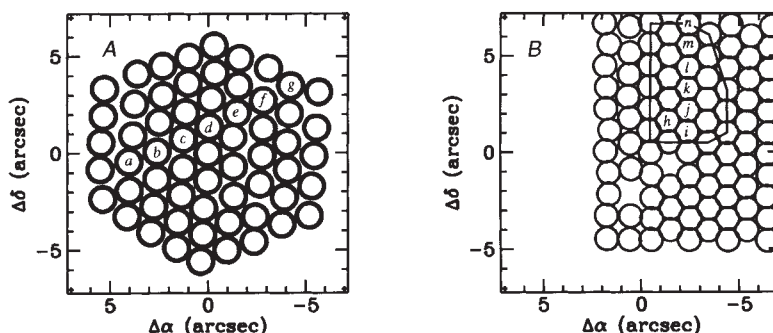
The optical velocities were obtained from the centroids of the gaussian fits to the H α , [N II] 6,548, 6,584 Å, and [S II] 6,716, 6,731 Å, narrow emission lines at the position of the continuum maximum. Because of the asymmetrical shape of these lines, the velocity determination depends on the method used. Therefore, we have included from the literature a C80 (centroid at 80% of the maximum) value in one case¹⁶, and an average between the C50 and peak velocities in the other¹⁸. The average velocities are: $\langle v_{\text{opt}} \rangle = 1,079 \pm 18$ km s⁻¹, $\langle v_{\text{sys}} \rangle = 1,114 \pm 7$ km s⁻¹. The value between parentheses has been excluded in the computation of $\langle v_{\text{sys}} \rangle$.

galaxy. A satisfactory physical and dynamical description of the circumnuclear regions of Seyfert galaxies is, however, still lacking.

The data presented here were obtained on 8 May 1989 and 4 December 1992 at the Observatorio del Roque de los Muchachos on the island of La Palma. During both nights NGC3227 was in a high-activity state, with the broad emission lines clearly present. Two fibre-optic systems (Fig. 1) were used in combination with the 4.2-m William Herschel telescope to make simultaneous spectroscopic measurements of different regions of NGC3227 (Fig. 2). This technique^{6,7} permits the direct comparison of two-dimensional spectral features (for example, velocity fields and intensity maps; Fig. 3). Therefore, the position of the region showing the broad emission lines can be fixed unequivocally in the velocity field of the galaxy, as derived from the narrow emission lines. Figure 3a shows a displacement between the kinematical centre and the position of the BLR of about $\Delta\alpha = -2.7$ arcsec, and $\Delta\delta = 2.1$ arcsec. These offsets correspond to a projected distance of about 250 pc, adopting $H_0 = 75$ km s⁻¹ Mpc⁻¹ and a systemic velocity $v_{\text{sys}} = 1,144$ km s⁻¹. The BLR coincides with the intensity maxima of the H α , [N II] and [S II] narrow emission lines, as well as with the intensity peak of the optical continuum (6,400–6,450 Å) within an estimated uncertainty of ± 0.3 arcsec. A spatial coincidence of the radio peak and the optical nucleus within ± 0.3 arcsec has also been reported^{8,9}.

In addition to the spatial separation there is also a shift between the NEL radial velocities at the position of the optical continuum maximum ($v_{\text{opt}} = 1,085$ km s⁻¹) and the kinematical centre ($v_{\text{kin}} = 1,136$ km s⁻¹), which is corroborated by the results of other authors (see Table 1). In fact, the average value of previous systemic velocity determinations ($\langle v_{\text{sys}} \rangle = 1,144$ km s⁻¹) agrees with the value of v_{kin} derived by us. This

FIG. 1 The distribution of the optical fibre faces at the telescope's focal plane in 1989 (A) and in 1992 (B). The white circles represent the core (transmitting area) of the fibres, and the surrounding black rings their claddings. The core and cladding diameters are, respectively, 0.9 and 1.2 arcsec for the 1989 bundle, and 0.9 and 1.1 arcsec for the 1992 one. Units are presented in sky coordinates relative to the focal plane scale of the William Herschel telescope. The 1992 bundle has been shifted with respect to the 1989 one to normalize their relative positions on the sky during the observations, using as reference the NGC3227 continuum maximum. At the other end of each bundle, the faces of the fibres form a linear array ('pseudo-slit') directing the light into a grating spectrograph, which is able to record simultaneously the spectra from all the fibres.



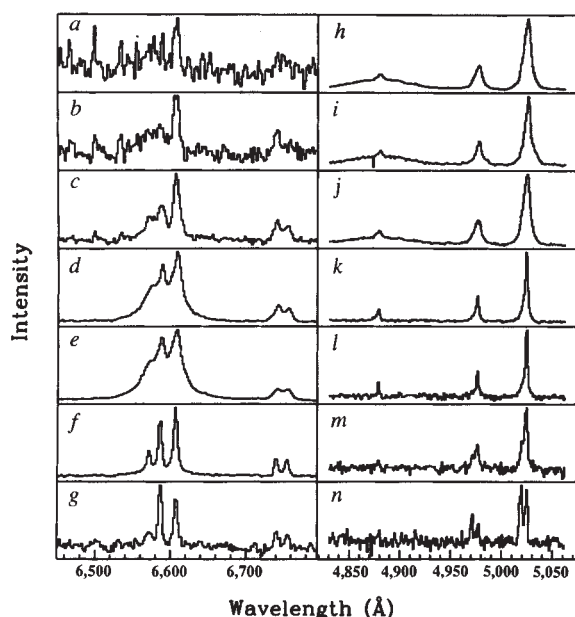


FIG. 2 Typical spectra from NGC3227, corresponding to the 1989 (a–g) and 1992 (h–n) observing periods. The letters indicate the fibres from which the spectra came. For the 1989 data the spectral resolution was 3.3 Å in a band from 6,300 to 7,050 Å (somewhat larger than displayed). The signal-to-noise (S/N) ratio for the data in this period was good enough to permit gaussian fitting of the line profiles for nearly all the spectra. The 1989 spectra presented here correspond to the fibres aligned with position angle 110° passing through the optical nucleus (e) and very near to the kinematical centre (c). In 1992 two spectral regions, 4,460–5,265 Å and 6,040–6,959 Å, were simultaneously observed with spectral resolution of ~ 2 Å. In this case the S/N ratio was lower and gaussian fitting was possible only for spectra from near the optical nucleus (h). The optical fibres were distributed as indicated in Fig. 1B. (The spectral range displayed here is 4,830–5,062 Å.) Evidence for two components in the [O III] lines were observed in a region from the optical nucleus to the North, the clearest case being spectrum (n), where two resolved peaks are observed. This suggested gaussian decomposition of the [O III] profiles into two components, which could be discriminated in terms of their mean parameters. In particular it was found that the broader component ($\langle \text{FWHM} \rangle = 9.1 \pm 3.6$) was generally blueshifted with respect to the other component ($\langle \text{FWHM} \rangle = 3.0 \pm 1.7$). The narrower (red) component seems to be linked to $\text{H}\beta$, as they have similar widths and they give the same radial velocities. The $\text{H}\beta$ counterparts of the blue component are very weak in our spectra. This supports the idea that the red component (narrower and of lower excitation) is representative of the mean galactic behaviour, while the blue component seems to be more related to the active nucleus itself.

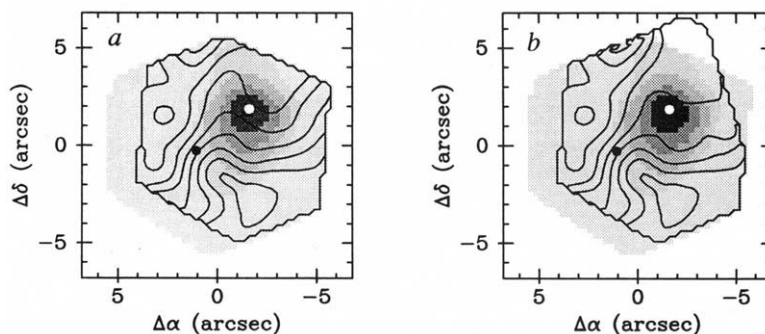
supports the argument that the kinematical centre actually represents the galactic mass-centroid. The average velocity derived from the NLR ($\langle v_{\text{opt}} \rangle = 1,079 \text{ km s}^{-1}$) is also in good agreement with our determination of v_{opt} .

The velocity field presented in Fig. 3a shows a pronounced distortion of the isovelocity contours in the neighbourhood of the BLR, which seems to arise from a blueshift relative to the systemic velocity. This confuses the interpretation of the observed velocity pattern, and could have some kinematical significance. The blueshift of the optical nucleus, also present in other Seyfert galaxies, may arise from some complex kinematics hidden by the line profiles. To analyse this question we have examined the [O III] 5,007-Å, 4,959-Å and $\text{H}\beta$ lines (see Fig. 2). The twin [O III] lines seen in Fig. 2n suggest that the [O III] lines in the blueshifted region can be decomposed into two components. By doing so, we have obtained a corrected velocity field

(Fig. 3b), using the velocities derived from the component that better fits the general galactic behaviour in the region affected by the blueshift. The velocity gradient across the optical nucleus is very smooth in this map, which may represent a nearly unperturbed rotational velocity field. This suggests that the distortions are related to the nuclear activity itself, supporting the determination of the kinematical centre and, consequently, the off-centre location of the BLR. The blueshifted component could represent either radial gas outflow from the active nucleus, or motion of the BLR disconnected from the mean galactic motion. It is of great importance to derive the velocity field from stellar absorption lines (for example, the calcium triplet), although the relatively faint continuum near the kinematical centre makes this difficult with the current observational techniques.

Models of barred spiral galaxies also predict the presence of non-circular motions and in principle it should be possible to

FIG. 3 Direct (a) and corrected (b) velocity fields (contours) are superimposed on the continuum (6,400–6,450 Å) intensity map (grey-scale plots) of NGC3227. The continuum map was corrected for the difference in sensitivity between fibres (and detector pixels) using an exposure of uniform source (a blank area of the sky during twilight). Note that while the continuum can be determined over the entire area covered by the hexagonal bundle, reliable velocity determinations are restricted to a smaller region. We have plotted ten isovelocity lines between 930 and 1,330 km s^{-1} . The northeast (top right) region is moving towards us. The intensity step between two consecutive logarithmically scaled grey levels is ~ 2 (excluding the first level which was selected arbitrarily to fill all the area covered by the bundle). The direct velocity map was determined from simultaneous gaussian fitting to the $\text{H}\alpha$, [N II] 6,548, 6,584 Å, and [S II] 6,716, 6,731 Å emission lines. The velocity uncertainty is estimated to be about $\pm 15 \text{ km s}^{-1}$. In panel a the pattern of a typical galactic velocity field can be clearly recognized towards the southeast of the continuum maximum, with the major kinematical axis separating the two poles. The black dot indicates the approximate position of the kinematical centre, determined from the gradient of the velocity field. The heliocentric radial velocity of the centre is $1,136 \text{ km s}^{-1}$. This figure clearly shows the displacement between the kinematical centre and the continuum maximum (which is nearly coincident with the BLR). The corrected map (b) has been generated by considering the velocity determinations corresponding to the $\text{H}\beta$ and red



(galactic) component of the [O III] 4,959, 5,007 Å lines (see text and Fig. 2) in the region $\Delta\delta > 0.5$, $\Delta\alpha < -0.5$ arcsec, whereas in the remaining region, the same values as in (a) were used (see Fig. 1). In this map the velocity gradient across the optical nucleus is smoother and more regular. Straight lines through the velocity field, crossing the kinematical centre and aligned with position angles 145° and 55°, define quite well the kinematical major and minor axes, respectively. The change in projected velocities along position angle 145° is in agreement with that observed in other galaxies, going from -140 to 160 km s^{-1} . Along position angle 55° the velocity changes are small ($\pm 30 \text{ km s}^{-1}$). This supports the determination of the kinematical centre and the result that the corrected velocity field is due mainly to rotation.

obtain an alternative explanation to the kinematical disturbance from a detailed and specific gas flow-model incorporating the influence of the bar (or the interaction with the companion). The presence of a bar, however, is not established, and infrared emission from dust (possibly associated with the bar) is concentrated in the galaxy disk, and is therefore not directly related to the nucleus¹⁰.

There are a few other cases of active galaxies with maxima of optical continua and/or narrow emission line that are displaced, in velocity and space, from the kinematical centre^{11–14}. In the present case, it is the BLR itself (in addition to the maxima of the continuum and the narrow emission lines) that is found at considerable distance from the kinematical centre of NGC3227. Arguments against an interpretation of the results in terms of an off-centre Seyfert 1 nucleus include: (1) the offset of the BLR is only with respect to the velocity field derived from the motions of the ionized gas which might not represent the true galactic velocity field; (2) the apparent displacement is attributed to dust obscuring the light, and hence affecting either the velocity field or the 'true' position of the nucleus; and (3) the direct observation of light coming from the 'true' nucleus is blocked by obscuring matter and we are receiving indirect fluorescent or scattered radiation. We believe, however, that none of these possibilities is satisfactory if one takes into account the symmetry of the velocity field in the vicinity of the kinematical centre, the agreement between v_{sys} and v_{kin} , the coincidence of the optical and radio peaks, and the short-term variability of the broad emission lines.

In the standard model of active galactic nuclei the collapse of a superdense star cluster leads to the supermassive black hole which is supposed to be at the centre of the BLR. The formation and presence of a black hole orbiting a centre of relatively low luminosity could imply the existence of a large quantity of dark matter. Alternatively, the black hole could be created at the kinematical centre and subsequently migrate to its present position. This possibility is supported by some theoretical models¹⁵, which predict that shifts in the location of the centre of the gravitational potential could push a particle situated at the nucleus around the mass centroid.

Another possible interpretation of the observed behaviour is the coexistence of two nuclei, resulting from the merger of two galaxies¹². The main drawback of this hypothesis as applied to NGC3227, is the lack of evidence in the optical continuum map for a second nucleus. It is worth noting, however, that there is evidence for a secondary weak radio emission peak near the position of the kinematical centre⁸. □

Two-electron quantization of the charge on a superconductor

P. Lafarge, P. Joyez, D. Esteve, C. Urbina & M. H. Devoret

Service de Physique de l'Etat Condensé, CEA-Saclay, F-91191 Gif-sur-Yvette, France

THE theoretical understanding of superconductors is based on the notion of electron pairing into Cooper pairs¹. The first direct evidence for electron pairing was the observation that the flux threading a superconducting ring is always a multiple of the flux quantum, given by the ratio of Planck's constant to the Cooper-pair charge $2e$ (refs 2, 3). Here we report a direct measurement of the total charge on a superconducting electrode which is free to exchange electrons with a metallic reservoir through a tunnel junction. The total charge on a non-superconducting metal electrode has been shown previously⁴ to increase in jumps of $1e$, corresponding to the addition of single electrons. We have also observed steps of $1e$, with an even-odd asymmetry, for a superconducting electrode when the charging energy exceeds the energy gap between the ground and first excited superconducting state⁵. Our present measurements, with the charging energy below the gap, reveal charging steps strictly quantized in units of $2e$, corresponding to the simultaneous tunnelling of two electrons. The $2e$ steps break into $1e$ steps when the temperature and magnetic field are increased above threshold values, corresponding to the electrostatic breaking of a single Cooper pair. Our results indicate that Cooper pairs can be manipulated in the same way as single electrons in turnstile and pump devices⁶.

Figure 1 shows a schematic diagram of the experiment. A $\text{Cu-Al}_2\text{O}_3\text{-Al}$ tunnel junction of capacitance C_j in series with a

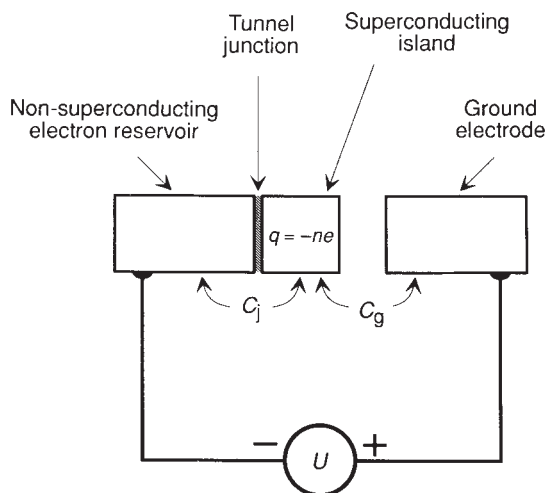


FIG. 1 Schematic diagram of the experiment. The superconducting island is a $30 \times 110 \times 2,260$ nm Al strip containing $\sim 10^9$ atoms. Its dimensions are such that the electrostatic energy of one extra electron is much larger than the energy $k_B T$ of thermal fluctuations at temperature $T \sim 30$ mK. The island can exchange electrons with a Cu (3 wt% Al) thin-film electrode (which acts as an electron reservoir) through a tunnel junction¹⁷. The total charge q of the island varies under the influence of the externally controlled voltage source U connected between the electron reservoir and a ground electrode. The variation with U of the time average $\bar{q}$ of the island charge is measured by a Coulomb blockade electrometer (not shown) which is weakly capacitively coupled to the island. The nanofabrication and low-noise measurement techniques involved in this type of experiment have been described in refs 5 and 18.

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capacitor C_g is biased by a voltage source U . The aluminium electrode which is common to both the junction and the capacitor, the 'island', is surrounded by insulating material. Because the junction tunnel resistance R_t is such that $R_t \gg R_K = h/e^2$, the total charge q of the island is a good quantum number and is given by $q = -ne$ (ref. 6). As U increases, electrons will tend to move into the island to minimize the total energy of the circuit, which is the sum of its electrostatic energy and of the internal energy of the island⁷. The fluctuations of n are determined by the ratio between the energy of thermal fluctuations and the Coulomb energy $E_c = e^2/2(C_j + C_g)$, which is the electrostatic energy cost of putting one extra electron on the island when $U=0$. By nanofabricating the circuit of Fig. 1, E_c can be made of the order of 2 K. By lowering the circuit temperature to ~ 30 mK, we can ensure that n has negligible fluctuations and adopts the minimum energy value. This is demonstrated by the following control experiment. We placed the island in the non-superconducting state by applying a magnetic field (0.2 T) and measured the variations of the time-averaged charge $\bar{q}$ with voltage U , using a Coulomb blockade electrometer⁸ operated in a feedback mode. The data are shown in Fig. 2a. If q was not quantized, the circuit would achieve an equilibrium charge configuration with no potential difference on the junction capacitance C_j and hence, $\bar{q} = C_g U$. Because q is quantized, $\bar{q}$ can only increase in steps, which are located at half-integer values of the reduced voltage $C_g U/e$.

We then placed the island in the superconducting state by suppressing the magnetic field. The results are shown in Fig. 2c. There is again a stepwise variation of $\bar{q}$ versus U , but the height and length of the steps have doubled, indicating that only electron pairs are transferred from the reservoir into the island. When we applied an intermediate magnetic field, so as to reduce substantially the superconducting gap without suppressing superconductivity, we observed an intermediate staircase pattern that consisted of a succession of long and short e -steps (Fig. 2b). This was similar to the pattern observed in a previous experiment involving a different sample⁵. The ratio between the length of the short (S) and long (L) steps was observed to decrease as we lowered the field. Below a threshold field $H=0.02$ T, the short steps disappeared completely and perfect $2e$ -quantization was recovered.

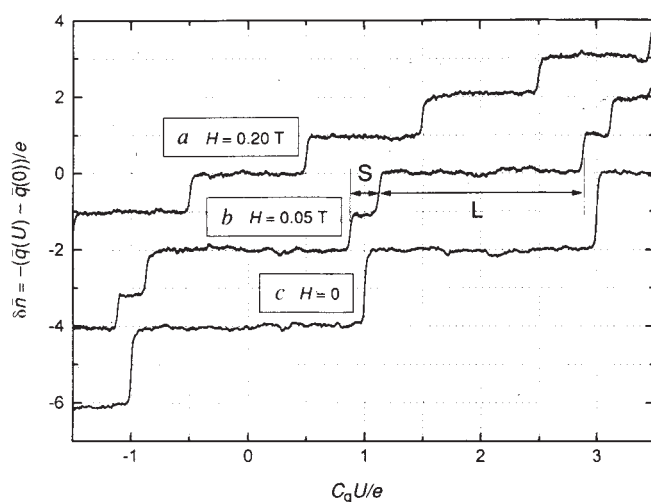


FIG. 2 Variations of the average value $\bar{q}$, in units of e , with the polarization $C_g U/e$, at $T = 28$ mK, for three values of the magnetic field H applied to the sample. *a*, Non-superconducting island. *b* and *c*, Superconducting island. For clarity, *b* and *c* have been offset vertically by 2 and 4 units, respectively. The letters L and S refer to the long and short steps, respectively.

These results can be understood by considering the total free energy of the circuit: $E = E_c(n - C_g U/e)^2 + (n \bmod 2)\bar{\Delta}$ + terms independent of n . The first term is simply the electrostatic energy of the circuit, that is the electrostatic energy of C_j and C_g and the work of the voltage source U (ref. 7). The second term is the island internal energy which depends on n only through its parity⁹, the parameter $\bar{\Delta}$ denoting the odd-even free energy difference¹⁰. Such an odd-even difference is expected for a superconductor, because given an odd number of electrons, one of them cannot be paired and must remain as a quasiparticle excitation the energy cost of which is the superconducting energy gap. From this model we can predict the ensemble average $\langle n \rangle$ which we suppose equal to the temporal average $\bar{n}$ measured in

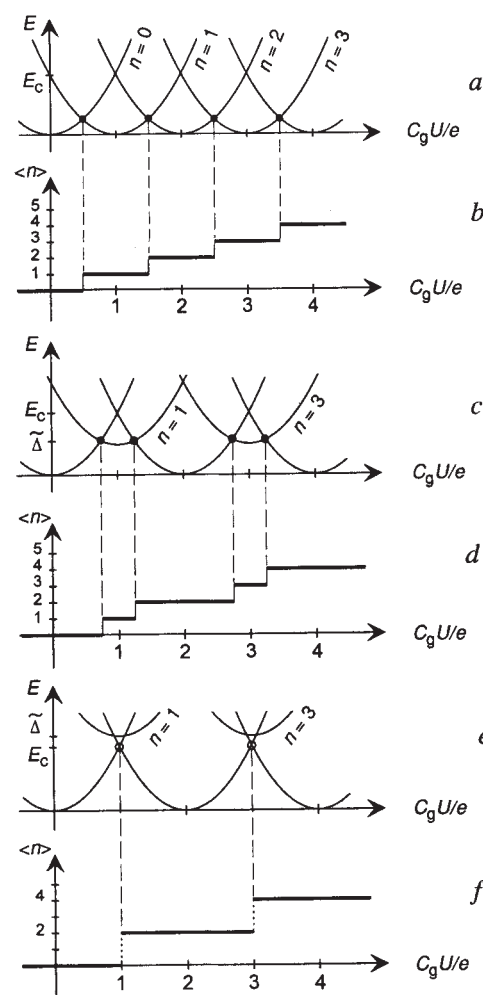


FIG. 3 Total energy of the circuit of Fig. 1 as a function of the polarization $C_g U/e$, for several values of the excess number n of electrons in the island, in the non-superconducting state (*a*) and superconducting state (*c*, *e*). E_c is the electrostatic energy of one excess electron on the island for $U=0$. The minimum energy for odd n is $\bar{\Delta}$ above the minimum energy for even n . Panels *c* and *e* differ by the relative magnitude of $\bar{\Delta}$ and E_c . The black dots correspond to level crossings where a single electron tunnels into and out of the island. The hollow circles correspond to level crossings where the only allowed process is the simultaneous tunnelling of two electrons into the island to form a pair (Andreev process). The equilibrium value $\langle n \rangle$ versus $C_g U/e$ is shown in the non-superconducting (*b*) and superconducting (*d*, *f*) states, at $T=0$. The Andreev process is shown in *f* by a vertical dashed line to distinguish it from the single electron tunnelling process shown in *b* and *d* by a vertical continuous line.

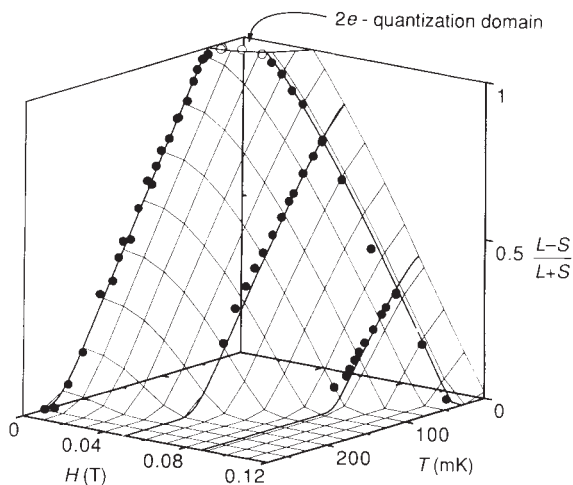


FIG. 4 Odd-even step-length ratio, plotted as $(L - S)/(L + S)$, as a function of the temperature T and magnetic field H . The fully $2e$ -quantized steps are shown as hollow circles with unit height. The black dots are such that $1 > (L - S)/(L + S) = \tilde{\Delta}/E_c$. The surface defined by the grid is the theoretical prediction combining references 5, 10, 15 and 16. Note that the $2e$ -quantization domain is only a small portion of the odd-even asymmetry domain ($\tilde{\Delta} > 0$) which is itself a small part of the superconductivity domain ($\Delta > 0$).

the experiment. In Fig. 3a we show, as a function of U , the energy of the different n states for the non-superconducting case $\tilde{\Delta} = 0$. At temperatures T such that $k_B T \ll E_c$, n will adopt the value of the integer closest to $C_g U/e$, which corresponds to the lowest energy state, hence the staircase pattern of Fig. 3b. In Fig. 3e we show the case of a superconducting island such that, at the lowest temperatures, $\tilde{\Delta} > E_c$ in zero magnetic field. In that case, for every value of U , the ground state of the circuit always corresponds to an even n , which explains the doubling in Fig. 3f of the step height compared to Fig. 3b. The energy asymmetry between states with even and odd n has recently been observed through the $2e$ -periodicity of the gate-charge dependence of the current in Coulomb blockade electrometers with a superconducting island^{10, 12} and of the asymmetric e -staircase of a superconducting box⁵. It is important to note that although $2e$ -quantization implies necessarily $2e$ -periodicity, the converse is not true, as shown by Fig. 3d. The results reported here reveal new information: direct transitions between fully paired even states, which do not create a quasiparticle excitation, can be the sole charge-transfer mechanism, provided that $\tilde{\Delta} > E_c \gg k_B T$, conditions which could not be satisfied in previous island-charge measurements. This perfect $2e$ -quantization necessitates that the system finds, as U is increased, its lowest energy state by the coherent tunnelling of two electrons from the reservoir into the island to form a Cooper pair. The rate of this process, also known as Andreev reflection¹³ is proportional to $(R_K/R_t)^2$ (ref. 14) and is therefore much weaker than single-electron tunnelling, the rate of which is proportional to R_K/R_t . Nevertheless, because the $2e$ -steps of Fig. 2c did not display any measurable out-of-equilibrium behaviour, the timescale of the Andreev process is shorter than our measurement timescale which is of the order of 10^{-2} s.

At intermediate magnetic fields and temperatures (Fig. 3c), the odd-even free-energy difference, although non-zero, is such that $\tilde{\Delta} < E_c$. Odd- n states can now exist in a finite U range (Fig. 3d). In this regime, we can measure $\tilde{\Delta}/E_c$ from the length ratio S/L of the short and long steps. This can be done quite accurately because the sharpness of the steps makes S/L insensitive to the long-term drift in the electrometer output due to offset charges⁷. The measurement of $(L - S)/(L + S) = \tilde{\Delta}/E_c$ with temperature and magnetic field, which was applied perpendicularly to the strip, is shown in Fig. 4. At a fixed magnetic field, we found that we could fit the measured $\tilde{\Delta}(T)/E_c$ using the theory of refs 5 and 9 with the quasiparticle density of states of Skalski *et al.*¹⁵, in which only one field-dependent parameter occurs, namely the pair-breaking energy Γ . The other parameter in this expression for the density of states is the zero-temperature zero-field energy gap Δ . Using the de Gennes and Tinkham prediction $\Gamma/\Delta = (\pi^3/18)H^2 d^2 \ell \xi_0/\Phi_0^2$ for a strip of dirty superconductor in a perpendicular field H (ref. 16), we derived a theoretical expression for $\tilde{\Delta}(T, H)/E_c$ which depends only on three parameters: the gap Δ , the Coulomb energy E_c and the elastic mean free path ℓ (in the expression for Γ , $d (= 110 \text{ nm})$ is the width of the strip, $\xi_0 (= 1600 \text{ nm})$ is the coherence length and Φ_0 is the flux quantum $h/2e$). The best fit, shown in Fig. 4, yields $\Delta/e = 210 \mu\text{V}$, $\Delta/E_c = 1.23$, values that are consistent with independent measurements, and $\ell = 6 \text{ nm}$. This latter value is one order of magnitude smaller than the mean free path we extracted from a conductivity measurement of a nanofabricated Al wire with same lateral dimensions as the island. This discrepancy may, however, simply reflect the fact that electron diffusion is not isotropic in the island; the main result shown in Fig. 4 is that $2e$ -quantization occurs in only a small portion of the superconductivity domain. □

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Does C₆₀ have a liquid phase?

M. H. J. Hagen*, E. J. Meijer*, G. C. A. M. Moolij*,
D. Frenkel*† & H. N. W. Lekkerkerker‡

* FOM Institute for Atomic and Molecular Physics, Kruislaan 407,
1098 SJ Amsterdam, The Netherlands

‡ van't Hoff Laboratory, Utrecht University, Padualaan 8,
3584 CH Utrecht, The Netherlands

ABOVE a substance's liquid–vapour critical point (T_c), the distinction between the liquid and vapour phases disappears. Below the triple point (T_t), meanwhile (at which solid, liquid and vapour coexist), only the solid and vapour are stable. The liquid range, T_c/T_t , depends on the nature of the intermolecular forces: for argon, $T_c/T_t = 1.8$, whereas for sodium the ratio is 7.5. But might there be molecular substances that have no liquid phase at all? Here we present results which suggest that C₆₀ is such a substance. We map out the phase diagram using computer simulations in which the C₆₀ molecules are represented by spheres interacting via Lennard-Jones potentials summed over all 60 carbon atoms. We find that the sublimation line passes above the metastable liquid–vapour coexistence curve. By drawing an analogy with the aggregation of colloidal particles, we expect that solid C₆₀ formed by nucleation from the vapour phase will be amorphous rather than crystalline.

The phase behaviour of C₆₀ might be expected to differ qualitatively from that of simple fluids such as argon or methane. As is well known, the equations of state of such simple molecular systems obey the law of corresponding states. This means that if the temperature T , the pressure P and the density ρ are expressed in units of the critical temperature, pressure and density respectively, the equations of state of such fluids can (very nearly) be superimposed. The reason why the law of corresponding states works for these systems is that, to a good approximation, the pair potentials of noble gases and simple spherical molecules all have the same shape. A good approximation to the effective pair potential of noble gas atoms is the so-called Lennard-Jones 12–6 potential (Fig. 1). Although C₆₀ is also a fairly inert, nearly spherical molecule, its intermolecular potential is not Lennard-Jones-like. A simple pair potential that should describe the intermolecular interactions of C₆₀ at high temperatures, has recently been proposed by Girifalco¹. This potential was constructed by assuming that the carbon atoms on two different C₆₀ molecules interact through a simple Lennard-Jones potential. At high temperatures we may average these interactions over all relative orientations of the C₆₀ molecules, giving the spherically averaged potential shown in Fig. 1.

As may be seen in Fig. 1, the pair potential of C₆₀ differs significantly from the Lennard-Jones form. In particular, we see that the ratio of the width of the attractive well to the diameter of the repulsive core of the potential is much less for C₆₀ than for noble gases. This is because the carbon carbon interaction determines the range of the attractive well, whereas the diameter of the repulsive core is fixed by the size of the C₆₀ 'cage'. In this respect C₆₀ and *a fortiori* the larger spherical fullerenes differ qualitatively from all smaller molecules. We should not expect C₆₀ to obey the same law of corresponding states as the noble gases.

What is the effect of the short range of the intermolecular interaction in C₆₀? As there are no other molecular systems with a similarly short-ranged potential, we turn for comparison to supra-molecular fluids, namely colloidal suspensions (for a review, see ref. 2). Uncharged colloidal particles in a nonpolar, refractive-index-matched solvent behave effectively like hard spheres. If we add non-adsorbing polymer to such a suspension, the polymer induces an effective entropic attraction between the

colloidal particles. The range of this attraction is comparable to R_g , the radius of gyration of the polymer. If R_g is larger than about one third of the radius of the colloidal particles, the phase diagram of the colloids includes a dense and a dilute disordered phase ('liquid' and 'vapour'), in addition to a colloidal crystal ('solid'). However, for shorter-ranged polymer-induced interactions, the 'liquid' phase disappears. In view of the short range of the pair potential shown in Fig. 1, it is natural to ask if something similar happens for C₆₀.

The phase behaviour of solid C₆₀ has been studied extensively, both at low³ and high⁴ temperatures. But there are, to our knowledge, no experimental data on the melting line of C₆₀ in the temperature range of the critical point ($\sim 1,800$ K, see below). We therefore decided to predict this part of the phase diagram by computer simulation. In fact, such simulations require a combination of several techniques that we briefly describe below.

To study the liquid–vapour coexistence of C₆₀, we used the 'Gibbs-ensemble' technique⁵. In this scheme, Monte Carlo simulations of the liquid and vapour phase are performed in separate periodic boxes. The two boxes can exchange volume and mass. This ensures that the liquid and vapour phase reach thermodynamic equilibrium. Using the techniques described in ref. 6, we could estimate the critical temperature to be $1,798 \pm 10$ K. We checked that only 15 K above this temperature, both boxes contain the same supercritical fluid phase.

But the Gibbs-ensemble technique can not be used to study solid–fluid coexistence: the mass-exchange between the fluid and the solid phase requires the addition or removal of molecules in an otherwise perfect crystal and this would necessarily result in the creation of point defects. In order to compute the melting line of C₆₀, we therefore used another approach. From thermodynamics we know that the condition for two-phase equilibrium is that both phases have the same temperature, pressure and chemical potential. In our simulations we can impose the temperature and the pressure. The chemical potential, however, must be computed separately. To this end, we performed absolute free-energy calculations^{7,8} in which we computed the chemical potential of the (face-centred-cubic) solid and the fluid phase at several temperatures. This allowed us to locate several points on the melting line, both above and below the estimated liquid–vapour critical point. The complete melting line was determined

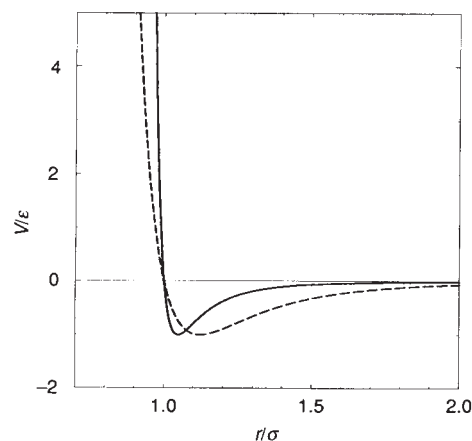


FIG. 1 A comparison of the 12–6 Lennard-Jones potential that describes the pair interaction of noble gases (dashed line), and the pair potential for C₆₀ proposed in ref. 1 (solid line). The potential energy V has been expressed in units of the well depth ϵ . For C₆₀, $\epsilon/k_B = 3,218$ K, where k_B is the Boltzmann constant. The intermolecular distance r is expressed in units of the effective diameter σ , defined as the distance where the pair potential crosses zero. For C₆₀, $\sigma = 0.959$ nm. In all simulations, we truncated the potential at $r = 2\sigma$ where the interaction energy is only 0.76% of the well depth.

† To whom correspondence should be addressed.

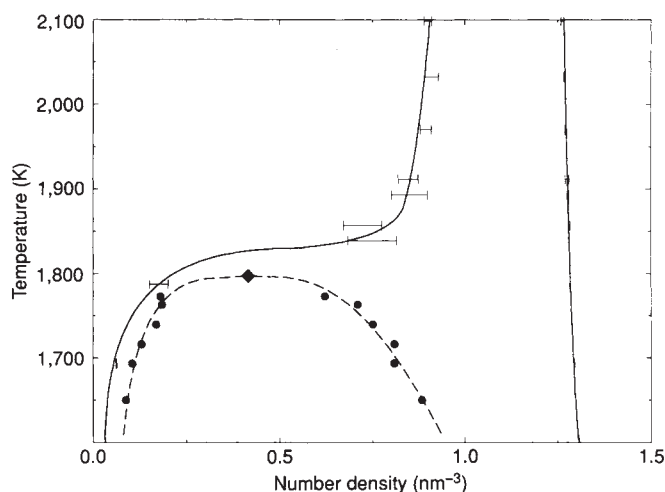


FIG. 2 Computed temperature-density phase diagram of C_{60} for temperatures in the region of 1,800 K. The solid lines are the solid-fluid coexistence lines. They were obtained by a fit to the data points (indicated by error bars) derived from free-energy calculations^{7,8} at 3,218, 1,893, 1,839, 1,788 and 1,694 K and 'Clausius-Clapeyron' integration⁹ between 3,218 and 1,893 K. The area between the two solid lines is a two-phase region where solid coexists with fluid. The dashed line denotes the (metastable) liquid-vapour coexistence line. It was obtained by a fit to the data points (solid circles) that were computed using the Gibbs-ensemble simulation technique of ref. 5. The solid diamond indicates the estimated location of the critical point. The fact that the critical point is located below the solid-fluid coexistence line implies that C_{60} has no liquid phase.

by integrating the Clausius-Clapeyron equation, following the scheme developed by Kofke⁹.

Figure 2 shows our prediction for the phase diagram of C_{60} . The sublimation line passes 35 ± 10 K above the liquid-vapour critical point. All phases that occur below the sublimation line in Fig. 2 have a higher Gibbs free energy than the coexisting solid and vapour phases. It may be possible to reach the liquid-vapour coexistence curve by supercooling the fluid below the sublimation line. But the liquid phase that would then form is necessarily less stable than the coexisting solid and vapour phases. Our results therefore suggest that C_{60} has no stable liquid phase. To our knowledge, this would be the first example of a pure substance that has no triple point. Of course, these findings assume that the interaction potential for C_{60} proposed in ref. 1 is accurate. An improved description of the intermolecular interactions would presumably result in small shifts of the predicted phase boundaries, but this will do little to change the highly anomalous appearance of the C_{60} phase diagram. Specifically, we expect the (metastable) critical point to remain very close to the sublimation line. This has interesting consequences for the formation of solid C_{60} from the vapour phase. If C_{60} is cooled below the sublimation line, it will probably not nucleate crystals from the vapour phase because, as soon as it is cooled below the liquid-vapour spinodal (that is, the line where the homogeneous fluid becomes absolutely unstable), metastable liquid-like aggregates will form. One might think that the formation of these aggregates would assist subsequent crystallization. However, experiments¹⁰ and simulations¹¹ on colloids with short-ranged attractions indicate that such aggregates do not crystallize but gelate to form rather open fractal structures. By analogy, we expect that solid C_{60} that is formed by homogeneous nucleation from the vapour phase will form an amorphous, rather than a crystalline phase. This may explain why the high-temperature synthesis of C_{60} in the gas phase often results in sooty deposits.

After we had completed this work, A. Cheng *et al.*¹² reported a similar simulation study of C_{60} in which the phase boundaries were located using a series of approximations. As a result of these approximations, a narrow liquid range was seen above 1,800 K. □

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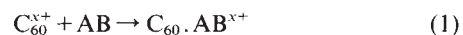
Enhanced reactivity of fullerene cations containing adjacent pentagons

Simon Petrie & Diethard K. Bohme

Department of Chemistry and Centre for Research in Earth and Space Science, York University, North York, Ontario, Canada M3J 1P3

GEOMETRICAL constraints, first identified by Euler, dictate that all of the closed carbon cages known as fullerenes must contain twelve pentagonal rings of carbon atoms¹. In all of the fullerenes synthesized so far, each pentagon is surrounded by hexagonal rings². Indeed, this has been proposed as a criterion for fullerene stability—the 'isolated-pentagon rule'^{1,3}—on the basis that adjacent pentagons are expected to be chemically reactive. Buckminsterfullerene (C_{60}) is the smallest fullerene for which the isolated-pentagon rule can be satisfied; smaller, adjacent-pentagon fullerenes have not been formed in bulk, but have been identified previously as cations^{4–6}. Here we report experimental evidence for the heightened chemical reactivity of cations of the adjacent-pentagon fullerenes C_{56} and C_{58} , relative to C_{60}^{x+} , which provides support for the basic assumptions underlying the isolated-pentagon rule. Our findings suggest that, if fullerenes such as C_{56} and C_{58} are produced as intermediates or byproducts of C_{60} generation either in the laboratory or in natural environments, they should form derivatives readily.

Using a selected-ion flow tube (SIFT) described elsewhere^{7,8}, we have observed many examples of addition reactions



for the ions C_{60}^{+} , C_{60}^{2+} and C_{60}^{3+} , and have determined that several factors influence the efficiency of reaction (1) for different charge states (x) and for different reactants (AB)^{9,10}. The ions C_{60}^{x+} are generated by the impact of electrons upon vaporized C_{60} , a technique which also produces ions C_n^{x+} ($n < 60$) by the dissociative ionization of buckminsterfullerene. The molecules chosen for reaction with these latter ions were several (CH_3CN , NH_3 , C_2H_4 , $n-C_4H_{10}$) which showed little reactivity with C_{60}^{x+} , because such reactions provide scope for observable rate enhancement.

The increased reactivity of C_{56}^{x+} and C_{58}^{x+} , compared to that of C_{60}^{x+} , can be readily seen from the kinetic measurements reported in Table 1 and illustrated in Figs 1 and 2. There is a considerable amount of experimental evidence to support the assumption that C_{56}^{x+} and C_{58}^{x+} are fullerene ions. For example, collisions of

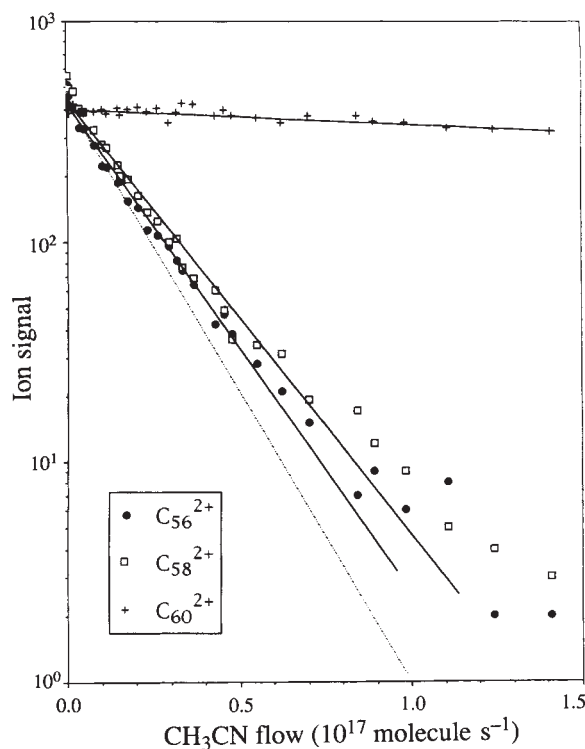
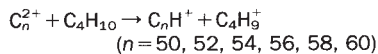


FIG. 1 Experimental data for the association reactions of C_{56}^{2+} , C_{58}^{2+} and C_{60}^{2+} with methyl cyanide, CH_3CN , performed at 294 ± 2 K and at a helium buffer gas pressure of 0.344 ± 0.001 torr. The exponential decay curves for C_{56}^{2+} and C_{58}^{2+} (which appear linear as the vertical-axis scale is logarithmic) are very much steeper than that observed for C_{60}^{2+} , indicating that the adjacent-pentagon fullerene ions C_{56}^{2+} and C_{58}^{2+} are much more reactive with CH_3CN than is C_{60}^{2+} . Rate coefficients for each ion are obtained from the least-squares line fitted to each ion signal plotted on such a graph. The corresponding ADO collision-rate coefficient is represented by the dotted line: clearly, C_{56}^{2+} and C_{58}^{2+} react with CH_3CN upon virtually every collision.

high-energy C_{60}^{x+} ($x=1-3$) and C_{58}^{x+} with helium result in formation of endohedral He adducts of the 'descendant' ions C_{60-2n}^{x+} and C_{58-2n}^{x+} , respectively: the incarceration of helium within the cavities of these descendant ions indicates that the surfaces have 'healed' rapidly to retain the fullerene structure^{11,12}. Further evidence is seen in the dissociation pattern for large carbon-cluster ions¹³—all even-carbon clusters larger than C_{32} lose only even numbers of carbon atoms on laser-induced fragmentation, whereas odd-carbon clusters in this size range lose an odd number of carbons. This is explained most readily by the dissociative model^{13,14} which presumes that the even-carbon cluster products containing ≥ 32 carbons are fully closed structures. In our own experiments, while we are able to generate substantial quantities of ions such as C_{50}^{x+} , C_{52}^{x+} , C_{54}^{x+} , C_{56}^{x+} and C_{58}^{x+} by dissociative ionization of C_{60} , we cannot produce detectable quantities of the neighbouring odd-carbon ions. This strongly suggests that the C_{60-2n}^{x+} fragment ions in our experiments are also fullerenes, and, by necessity, adjacent-pentagon fullerenes as they contain fewer than 60 atoms¹.

Of the four neutral reactants included in the present study, three react by association. The heightened efficiency of these reactions for the smaller fullerene ions C_{56}^{x+} and C_{58}^{x+} is especially significant because theory¹⁵ predicts that, in the absence of other factors, association efficiency should decrease with the decreasing size of the reactant ion or neutral; furthermore, this effect should be small for the reactions of such large ions with small neutrals. A rationale for the enhanced reactivity of C_{56}^{x+} and C_{58}^{x+} lies in the greater sp^3 character of carbons lying at the shared edge of adjacent pentagons, as shown in Fig. 3. The sp^3 character of the carbons within the reactant fullerene ion is important because each atom in a fullerene is already bonded to three others: formation of an additional bond between a fullerene carbon atom and an atom within a neutral reactant molecule requires tetrahedral coordination about this carbon atom, and this will be easier if this atom already possesses a degree of sp^3 character. All the carbon atoms in C_{60} , and in all higher isolated-pentagon fullerenes, possess largely sp^2 character: the increasing stability of fullerenes with increasing fullerene size¹⁶ is ascribed to the smoother, more graphite-like surface of the higher fullerenes. Inducing tetrahedral coordination at one carbon atom on

FIG. 2 Mass spectra obtained for a series of fullerene dications C_{50}^{2+} – C_{60}^{2+} , before (a) and after (b) addition of butane, C_4H_{10} , to the reaction vessel. The reaction observed for each dication is a hydride abstraction process:



The low reactivity of C_{60}^{2+} , in comparison to the other reactant ions, is apparent: under these conditions, C_{60}^{2+} (which is the most abundant reactant ion) produces less product ($C_{60}H^+$) than do all of the lower-mass, adjacent-pentagon fullerene dications.

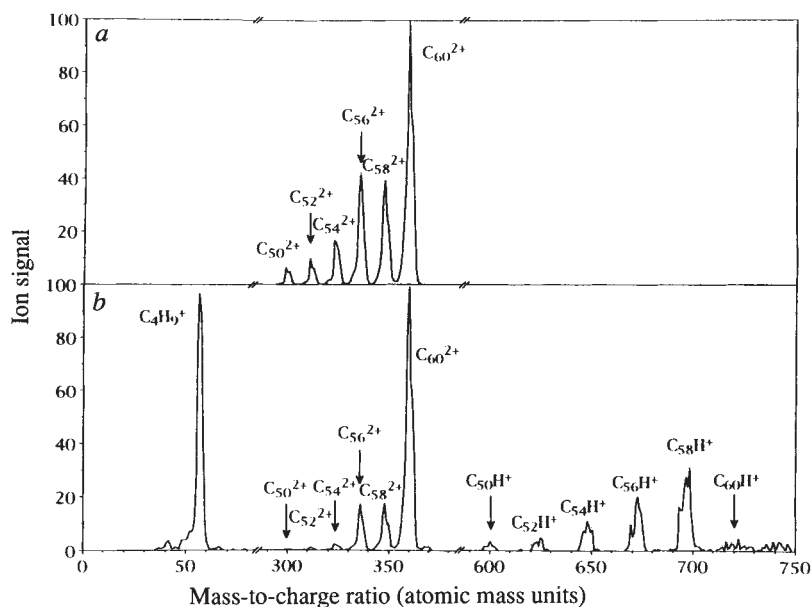
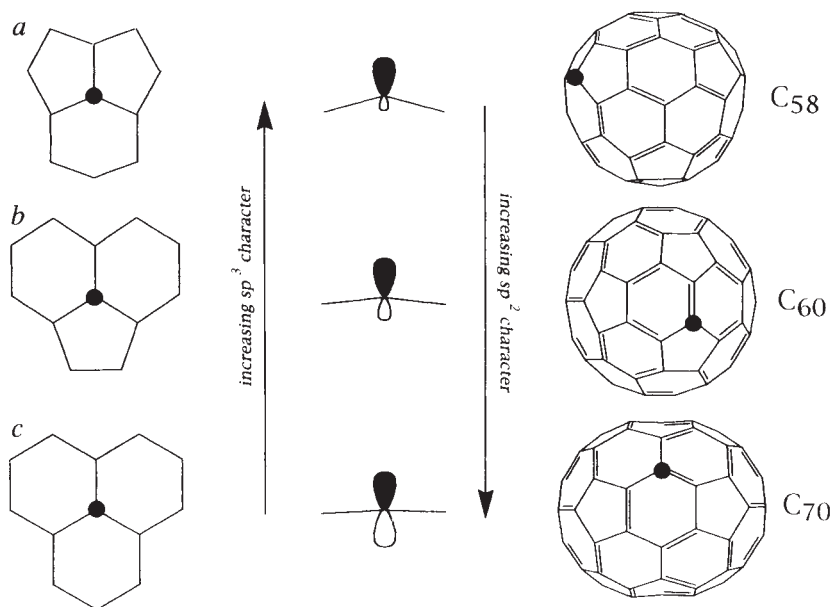


FIG. 3 An illustration of different environments for carbon atoms within fullerenes. Each carbon atom on the surface of a fullerene is involved in three pentagonal or hexagonal rings. Fullerenes smaller than C_{60} invariably possess structural features of type (a), involving two pentagons and one hexagon. These three rings are far from coplanar (as shown in the bond-and-orbital diagram beside (a)) and the orbitals of the central carbon atom are therefore expected to possess substantial sp^3 character. Structural features of type (b), the only feature found on the surface of C_{60} , involve two hexagons and one pentagon which are more nearly coplanar: the carbon atom involved here has bonds with a lower degree of sp^3 character. Fullerenes larger than C_{60} contain sites of type (b) but also possess structural features of type (c), which is also found in graphite and which has the lowest deviation from coplanarity of the three features discussed here. So, the bonding involved in this feature is expected to have a high degree of sp^2 character with little sp^3 character. The formation of a fourth chemical bond to a carbon atom, in a tetrahedral coordination, implies sp^3 hybridization at the carbon concerned in the resulting product. This process is expected to be easiest at sites which already feature a high degree of sp^3 character.



a surface such as that of C_{60} , which is constrained to remain as smooth as possible in order to maximize π overlap across the surface, is likely to be an energy-demanding process, and will only be feasible if the energy gain by means of bond formation is greater than the energy required to distort the carbon surface.

Although theoretical calculations have not, to our knowledge, predicted increased chemical reactivity for adjacent-pentagon fullerenes, they have indicated that such structures are substantially lower in thermodynamic stability than isolated-pentagon fullerenes. MNDO (modified neglect of diatomic overlap) calculations¹⁶ suggest a destabilization energy, per pair of adjacent pentagons, of about 7 kcal mol^{-1} , which is similar to the calculated resonance destabilization for the adjacent-pentagon conjugated hydrocarbon molecule pentalene. Calculations on C_{60} isomerism¹⁷ have indicated that a C_{60} structure with two

pairs of adjacent pentagons is $\sim 35 \text{ kcal mol}^{-1}$ higher in energy than the global minimum buckminsterfullerene structure.

A recent experimental study of high-energy collisions of the adjacent-pentagon fullerenes C_{58}^+ and C_{68}^+ with helium has demonstrated that, in contrast to similar experiments with C_{60}^+ and C_{70}^+ , the parent helium adduct is not formed in detectable quantities, although 'descendant' adducts (involving loss of C_2 units) are observed in all cases^{11,12}. The failure of the adjacent-pentagon adducts to remain intact has been attributed to the relative destabilization caused by the adjacent-pentagon structural feature¹².

Our results may have implications for chemical reactions of fullerenes in interstellar clouds and circumstellar shells. It has been suggested that fullerenes may be formed in the envelopes surrounding giant carbon-rich stars^{18,19} especially those with very low abundances of hydrogen. We have identified viable pathways to singly and doubly ionized fullerenes from neutral fullerenes under such conditions²⁰, indicating that ion-molecule reactions of these molecules may be significant in interstellar and circumstellar environments^{21,22}. Growth mechanisms for fullerenes are still a subject of debate: most proposed mechanisms involve the production of fullerenes from smaller neutral or ionized carbon fragments, although a recent study suggests that rearrangement and fragmentation of larger mono-, bi- and tri-cyclic carbon rings can also produce fullerenes²³. It seems possible that production of C_{60} involves the earlier, or simultaneous, formation of smaller fullerenes such as C_{58} and C_{56} . If this is the case, these smaller fullerenes will also be intermediates in, or byproducts of, the circumstellar generation of C_{60} . Our results suggest that those clusters which possess adjacent pentagons are likely to form derivatives readily in interstellar and circumstellar environments, which are expected to have spectral features distinct from those of the less-reactive fullerenes typified by C_{60} . □

TABLE 1 Reactions of C_n^{x+} ($x=56, 58, 60$) with various compounds

Reaction	Products	k_{obs}^*	$k_c^\dagger$	$k/k_{60}^\dagger$
$C_{56}^{2+} + \text{CH}_3\text{CN}$	$C_{56} \cdot \text{CH}_3\text{CN}^{2+}$	4.3	4.96	53.8
$C_{58}^{2+} + \text{CH}_3\text{CN}$	$C_{58} \cdot \text{CH}_3\text{CN}^{2+}$	4.2	4.94	52.5
$C_{60}^{2+} + \text{CH}_3\text{CN}$	$C_{60} \cdot \text{CH}_3\text{CN}^{2+}$	0.08§	4.93	1.0
$C_{56}^+ + \text{NH}_3$	$C_{56} \cdot \text{NH}_3^+$	0.0096	1.65	>9.6
$C_{58}^+ + \text{NH}_3$	$C_{58} \cdot \text{NH}_3^+$	0.034	1.65	>34.0
$C_{60}^+ + \text{NH}_3$	$C_{60} \cdot \text{NH}_3^+$	<0.001	1.65	1.0
$C_{56}^{2+} + \text{C}_2\text{H}_4$	$C_{56} \cdot \text{C}_2\text{H}_4^{2+}$	0.021	1.86	>21.0
$C_{58}^{2+} + \text{C}_2\text{H}_4$	$C_{58} \cdot \text{C}_2\text{H}_4^{2+}$	0.019	1.86	>19.0
$C_{60}^{2+} + \text{C}_2\text{H}_4$	$C_{60} \cdot \text{C}_2\text{H}_4^{2+}$	<0.001¶	1.86	1.0
$C_{56}^{2+} + n\text{-C}_4\text{H}_{10}$	$C_{56}\text{H}^+ + \text{C}_4\text{H}_9^+$	0.027	1.87	>27.0
$C_{58}^{2+} + n\text{-C}_4\text{H}_{10}$	$C_{58}\text{H}^+ + \text{C}_4\text{H}_9^+$	0.034	1.86	>34.0
$C_{60}^{2+} + n\text{-C}_4\text{H}_{10}$	$C_{60}\text{H}^+ + \text{C}_4\text{H}_9^+$	<0.001¶	1.86	1.0

* Observed effective bimolecular reaction rate coefficient (in units of $10^{-9} \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$) at $294 \pm 2 \text{ K}$ and $0.35 \pm 0.01 \text{ torr}$ of helium.

† Calculated ADO (Average-Dipole Orientation) collision rate coefficient (in units of $10^{-9} \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$) at 294 K , determined using the method of Su and Bowers²⁴.

‡ Rate enhancement observed for reaction, expressed as the ratio $k_{\text{obs}}(C_n^{x+}) : k_{\text{obs}}(C_{60}^{x+})$.

§ The reaction of C_{60}^{2+} was previously reported in ref. 10.

¶ The reaction of C_{60}^{2+} was previously reported in ref. 25.

|| The reaction of C_{60}^{2+} was previously reported in ref. 9.

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Evidence for strong acidity of the molecular sieve cloverite

Tery L. Barr*, Jacek Klinowski*†, Heyong He*, Klaus Alberti*, Georg Müller† & Johannes A. Lercher†

* Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, UK

† Institut für physikalische Chemie und Christian Doppler Labor für Heterogene Katalyse, Technische Universität Wien, Getreidemarkt 9/156, A-1060 Wien, Austria

ZEOLITES derive their catalytic activity from the strong acidity of protons attached to the negatively charged aluminosilicate framework, which makes the materials excellent proton donors. Unlike zeolites, the aluminophosphate molecular sieves^{1,2} are built from alternating AlO_4^- and PO_4^+ tetrahedra and are thus electrically neutral. Much attention has therefore been devoted to the generation of Brønsted acidity in these materials by introducing heteroatoms, such as Si, Mg, Fe, Co or Zn, to produce negatively charged frameworks^{3–6}. Similar arguments apply to gallophosphate molecular sieves^{7–10}, of which cloverite^{9,10} is a remarkable example. This extra-large-pore material contains pore openings in the form of a four-leafed clover, defined by a ring of 20 gallium and phosphorus atoms, some of which are linked to terminal hydroxyl groups. Here we use NMR, X-ray photoelectron spectroscopy (ESCA) and infrared spectroscopy to show that the P–OH groups in cloverite are localized versions of those in solid phosphoric acid, H_3PO_4 . Cloverite is thus a strong Brønsted acid even though no heteroatoms are present in its framework.

Cloverite has a cubic structure, $a = 51.712 \text{ Å}$, with the unit-cell formula $[\text{Ga}_{768}\text{P}_{768}\text{O}_{2,976}(\text{OH})_{192}] \cdot 192\text{QF}$, where QF is quinuclidinium fluoride. Single-crystal structural determination⁹ shows that there are five crystallographic framework sites for both P and Ga. Pure, highly crystalline cloverite was prepared by the method of Estermann *et al.*⁹. The surface/near surface composition was determined by ESCA to a depth of $\sim 50 \text{ Å}$. The $\text{Ga}(3p_{3/2})/\text{P}(2p)$ intensity ratio (Fig. 1a) gives $\text{Ga}/\text{P} = 1.15$, indicating a slight excess of surface gallium, which is consistent with results from related molecular sieves¹¹. High-resolution ESCA spectra reveal detailed core-level peak structures for each element. The binding energies and linewidths are given in Table 1, along with those for related materials. The 2.7-eV linewidth

of the $\text{Ga}(3d)$ ESCA spectrum (Fig. 1b and Table 1) is typical of a distinct gallium species, GaPO_4 , in the cloverite framework.

The broad and asymmetric $\text{P}(2p)$ ESCA line in Fig. 1c signifies the presence of two different oxidic phosphorus-containing species. Deconvolution (Fig. 1d) shows that the binding energy of the more abundant P species is 134.1 eV and that of the less abundant species is $\sim 132.3 \text{ eV}$. Although the progressive creation of electron holes in the sample during an ESCA experiment affects the apparent value of the binding energies, a general argument^{12–15} allows us to draw conclusions about the initial chemical state from the measured binding energies of oxides. Thus one may assume that, during the formation of an oxide, the more electropositive the element the more negative the $\text{O}(1s)$ binding energy in its oxide. Further, it is known^{14,15} that formation of a mixed $\text{A}_x\text{M}_y\text{O}_z$ oxide from two or more simple A_mO_n and M_xO_y oxides will in most cases result in the enhanced covalency of the more covalent of the two metal–oxygen bonds and, conversely, the enhanced ionicity of the more ionic of the two. This manifests itself as a lowering of the binding energy of the more covalently bonded element, and an increase in binding energy of the more ionically bonded. The $\text{O}(1s)$ binding energy for the mixed oxide lies between that of the two simple oxides.

The more intense $\text{P}(2p)$ line represents $\sim 84\%$ of the total phosphorus. This means that the more abundant oxidation state of phosphorus must belong to the Ga–O–P unit in the cloverite framework. The binding energies give a clue as to the nature of the other phosphorus species ($\sim 16\%$). Because of the presence of 'terminal hydroxyl groups' pointing towards the centre of the 20-membered ring, Ga–O–H and P–O–H bonds are present as well as Ga–O–P bonds. The ESCA technique is very sensitive to the nature of M–O–H bonds, and the chemical shifts easily distinguish hydroxides from the M–O–M* bonds of the equivalent oxide^{14,17}. The M–O–H bonds in cloverite should therefore produce an easily distinguished set of peaks in the appropriate M spectrum. This type of shifted spectral feature is found in the $\text{P}(2p)$ spectrum in Fig. 1c, corresponding to P–O–H bonds.

Solid-state NMR and infrared spectroscopy support the above interpretation. The ^{31}P magic-angle-spinning (MAS) NMR spectrum shown in Fig. 2a reveals features not seen by Merrouche *et al.*¹⁰ It consists of a main peak at -11.2 p.p.m. with a shoulder at $\sim -12.6 \text{ p.p.m.}$, and a separate smaller line at -6.3 p.p.m. As the intensity ratio of the two lines is 6.84:1, the main peak must correspond to $\text{P}(\text{OGa})_4$ structural units, and that at -6.3 p.p.m. to P–OH phosphorus atoms. This ratio is very close to the 7:1 required by the structural determination⁹. The lower ratio of 5.25:1 detected by ESCA (corresponding to a larger population of P–OH groups) is due to the higher surface sensitivity of the technique¹². The fine structure of the main NMR peak is caused by the presence of four different phosphorus sites. The correct-

TABLE 1 ESCA binding energies and linewidths of cloverite and related materials

	Ga(3d) (eV)	P(2p) (eV)	O(1s) (eV)	Al(2p) (eV)	Reference
P–O–Ga in cloverite	21.6 (2.7)	134.1 (2.3) [0.84]	531.9 (2.5)	–	This letter
P–O–H in cloverite	–	132.3 (2.3) [0.16]	531.9 (2.5)	–	This letter
$\text{AlPO}_4 \cdot 8$	–	135.0 (2.4)	532.8 (2.2)	75.4 (2.4)	23
Na_2HPO_4	–	133.1	NA	–	16
Na_3PO_4	–	132.3	NA	–	16
Ga_2O_3	20.5	–	530.9	–	16
P_2O_5	–	135.2	533	–	16
Al_2O_3	–	–	531.0 (2.0)	74.0 (2.1)	12, 16

Selected binding energies and (in round brackets) linewidths $\pm 0.2 \text{ eV}$, referenced to $\text{C}(1s) = 284.6 \text{ eV}$ from the quinuclidinium $-\text{CH}_2$ groups. The justification for this method of removing charging shifts has been given previously^{12,16,22}. Relative intensities are given in square brackets. The total linewidth of the overlapping $\text{P}(2p)$ lines from P–O–Ga and P–O–H in cloverite is 3.0 eV. NA, not applicable.

† To whom correspondence should be addressed.

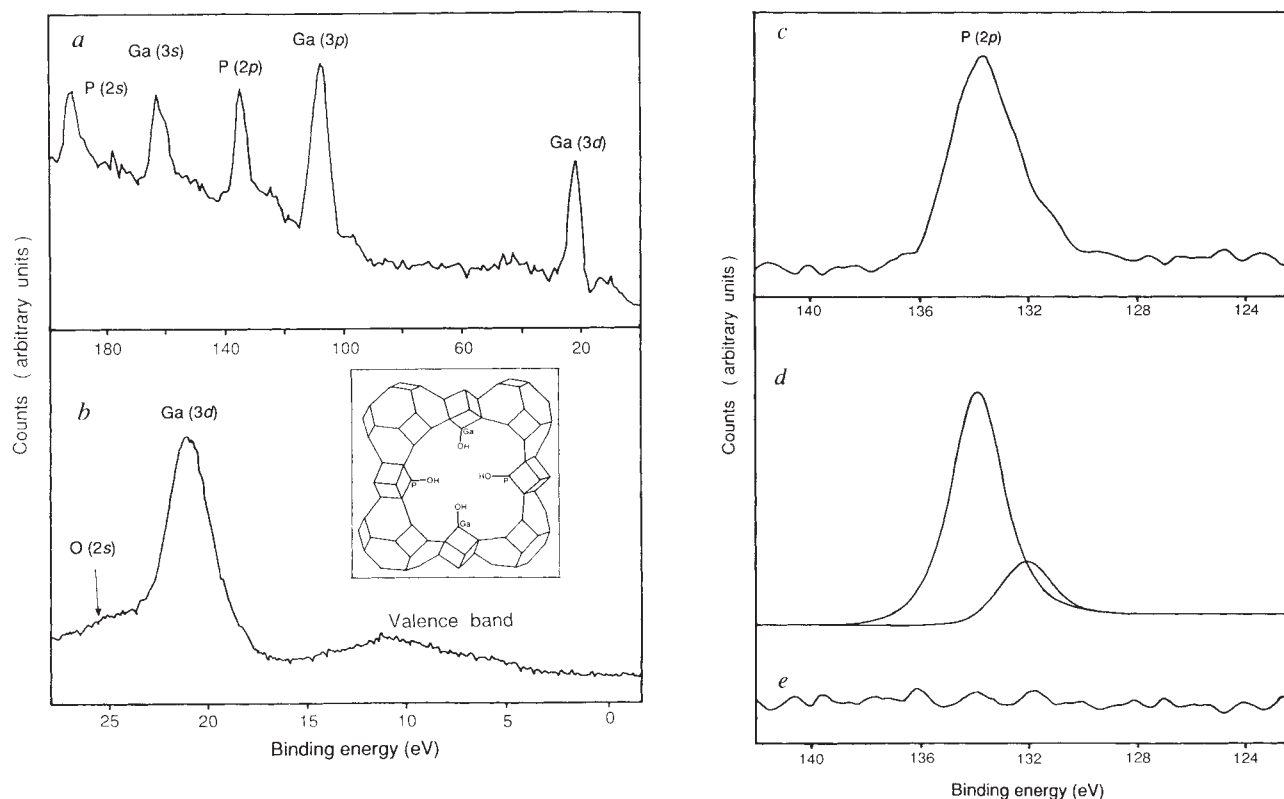


FIG. 1 ESCA spectra of as-prepared cloverite. *a*, Survey scan of the principal gallium and phosphorus peaks providing a quantitative elemental analysis. *b*, Scan of the valence region showing the Ga(3d) and O(2s) lines. *c*, The P(2p) spectrum. *d*, Computer deconvolution of P(2p) lines corresponding to phosphorus in different chemical environments: The difference between the deconvoluted and the experimental spectra

is given below (*e*). The spectra were acquired using a V.G. ESCALAB spectrometer at the Surface Analysis Facility of the University of Wisconsin-Milwaukee. The inset in *b* shows the shape of cloverite window, circumscribed by 20 Ga and P atoms, some of which are, usually, linked to terminal hydroxyl groups.

ness of our assignments is confirmed by the cross-polarization MAS NMR spectrum in Fig. 2*b*, which shows that the intensity of the peak at -6.3 p.p.m. is significantly increased upon cross-polarization. The P atom in question is therefore near a proton. The short optimum contact time of ~ 200 μ s indicates that the cross-polarizing proton belongs to the hydroxyl group directly bonded to the phosphorus, and not to the organic template.

It has been shown¹³⁻¹⁵ that the relative ionicity of a M-O bond increases as M comes from further and further down a given column of the periodic table. For example, Ga_2O_3 is more ionic than Al_2O_3 , and In_2O_3 more ionic than Ga_2O_3 ; the converse applies to the relative covalencies. Thus the P-O bond in Ga-O-P is substantially more covalent (P has a lower binding energy) than the P-O bond in the Al-O-P-containing materials, such as $\text{AlPO}_4\cdot 8$ (Table 1). The enhanced covalency of the P-O bond is the cause of the acidity of cloverite hydroxyls. In zeolites, the silica functions as the covalently bonded species, buffered by the relative ionicity of the Al-O bond of the AlO_4^- tetrahedron^{12,14,17}. Each aluminate unit requires the presence of a cation, and when that cation is H^+ a Brønsted-acid site results. Because the Al-O bond is itself moderately ionic, the corresponding O-H bond in simple systems containing Al-O-H (such as bayerite), is not very ionic, and indeed $\text{Al}(\text{OH})_3$ and AlOOH are amphoteric. On the other hand, when the aluminate ion is placed in the tetrahedral silica framework to form a zeolite, the acidity of the 'bridging' hydroxyl increases. This effect is amplified when all the Al-O-H bonds are surrounded by covalent Si-O bonds. Thus in zeolites with Si/Al ratios ≥ 2.5 , the ionicity of the O-H bond increases as the ratio becomes larger, and the zeolite becomes a solid acid. Unfortunately, as the strength of

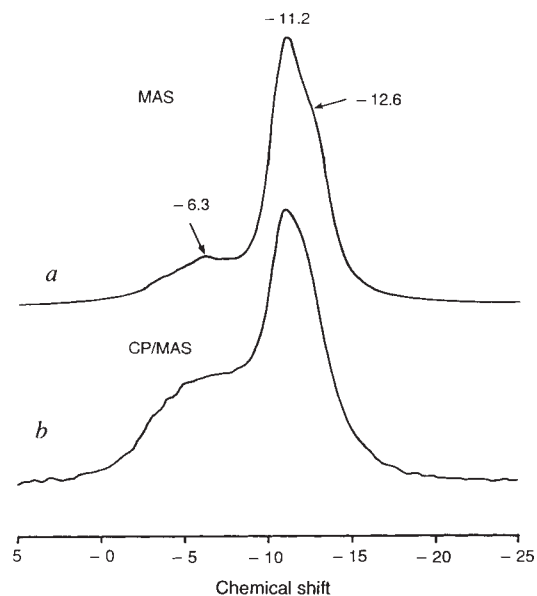


FIG. 2 ^{31}P NMR spectra of as-prepared cloverite. *a*, Magic-angle spinning (MAS) only. *b*, Cross-polarization magic-angle spinning (CP/MAS) with 200 μ s contact time. The spectra were measured at 161.8 MHz using a Chemagnetics CMX-400 spectrometer. Samples were spun at 4.5 kHz with air as the driving gas. CP/MAS spectra were acquired using variable contact times in the range of 100 μ s to 5 ms²⁴. The Hartmann-Hahn condition was established using a sample of NaH_2PO_4 . The chemical shifts are given in p.p.m. from external 85% H_3PO_4 .

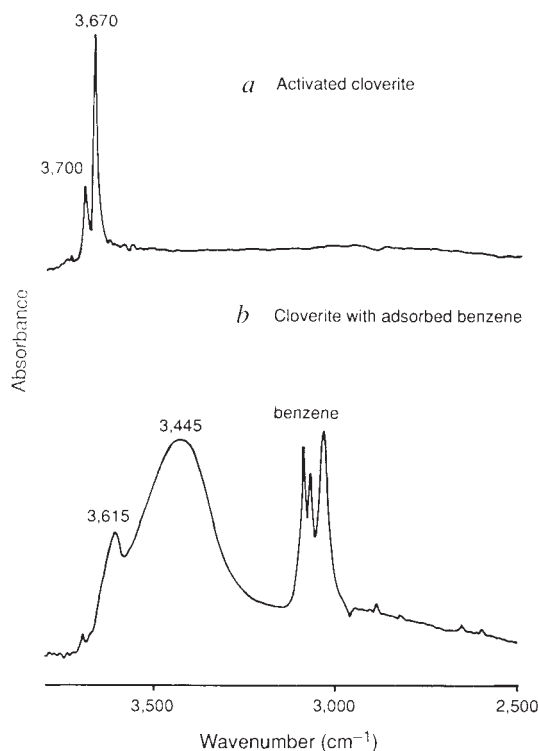


FIG. 3 Infrared spectra of a single crystal of cloverite. *a*, After activation at 800 K in synthetic air. *b*, After exposure to vapour (at a pressure of 1 mbar) at 300 K for 60 min. The measurements were carried out using an infrared microscope attached to a Bruker IFS88 spectrometer. Single crystals of cloverite, hexagonally shaped with a typical diameter of $\sim 80 \mu\text{m}$, were placed on a CaF_2 plate in the furnace-heated cell of an infrared microscope. They were activated by heating in flowing synthetic air (150 ml min^{-1}) at the rate of 10 K min^{-1} up to 800 K. This temperature was maintained for 90 min and then lowered rapidly to 300 K. The sample was then exposed to benzene vapour at 1 mbar for 60 min.

the acid sites in zeolites is increased, their concentration, determined by the Al content, falls.

In AlPO_4 and cloverite, the P promotes tetrahedral bonding and the dominant covalent lattice bond to oxygen. This means that the relative covalency of the P–O bond increases substantially on going from the Al–O bond of AlPO_4 to the more ionic Ga–O bond of cloverite (note the corresponding drop in the $\text{P}(2p)$ binding energy in Table 1). As a result, an O–H group also bonded to the phosphorus becomes even more ionic (that is, acidic) resulting in a solid phosphoric acid. This is reflected in the ESCA results, where the binding energy of the $\text{P}(2p)$ for the P–OH unit in cloverite is even lower than that of the P in Na_2HPO_4 (ref. 16) and the same as that for P–O–Na in Na_3PO_4 . This indicates an extreme ionicity of the O–H bond in this P–O–H group comparable to that for P–O–Na in Na_3PO_4 . Unlike zeolites, however, the number of acidic sites in cloverite does not decrease as the strength of those sites is increased. The positions of the acid sites are fixed, which enhances the selectivity of intracrystalline catalytic reactions. Also, acid sites in cloverite can not form hydrogen bonds which would reduce their strength, as is the case with phosphoric acids in solution¹⁸.

The type and acid strength of the hydroxyl groups in a single crystal of cloverite were investigated by infrared spectroscopy. After removal of the template by activation in dry air at 800 K, two narrow bands were observed at 3,670 and $3,700 \text{ cm}^{-1}$ (Fig. 3*a*), corresponding to two different kinds of hydroxyl groups. By analogy to the infrared spectra of supported H_3PO_4 (ref. 19), we attribute both hydroxyls to P–OH groups. Ga–OH groups correspond to substantially lower wavenumbers^{20,21}.

In general, the magnitude of the shift of the hydroxyl infrared bands on adsorption of a donor molecule is directly proportional to the acid strength of the OH group¹⁹. Upon adsorption of benzene (exposure (1 h) to benzene vapour at 1 mbar, corresponding to 1 benzene molecule per OH group), the OH stretching bands in cloverite shift from 3,700 to $3,615 \text{ cm}^{-1}$ and from 3,670 to $3,445 \text{ cm}^{-1}$ (Fig. 3*b*). These shifts, of 85 and 225 cm^{-1} , indicate low and high acid strength, respectively. For comparison, after adsorption of benzene on terminal Si–OH groups in silica and Si–OH–Al groups in zeolite H-ZSM-5, the hydroxyl

infrared bands shift to lower wavenumbers by 120 cm^{-1} and 260 cm^{-1} , respectively. We believe that the less-abundant weakly acidic hydroxyls in cloverite belong to terminal P–OH groups at the surface of the crystallite. All hydroxyl groups could be exchanged for OD groups with D_2O at 373 K in a few minutes.

The presence of heteroatoms in AlPO_4 and GaPO_4 molecular sieves is therefore not a prerequisite for the existence of Brønsted acidity. Materials containing bulk terminal P–OH units, of which cloverite is the only known example, may therefore be expected to be heterogeneous catalysts for a variety of reactions. The presence of P–OH in such sieves may be a rule rather than an exception. □

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rock, have light-REE-depleted patterns and La/Nb ratios comparable to those found in mid-ocean-ridge basalts (MORB), oceanic plateau basalts and some ocean island basalts (OIB; for example Icelandic picrites), but are distinct from island arc basalts (IAB). Other incompatible element ratios (Table 2) in the inclusions are, however, different from those in MORB and IAB (for example, K/Nb, Ba/La, Sr/Nd).

An issue that remains is whether the Belingwe komatiites are samples of ancient plume-related magmas or whether they are derived from the (Archaean) depleted mantle. If the latter, then these komatiites would be ancient analogues of mid-ocean-ridge magmas and our data would provide evidence for secular evolution in the composition of the depleted mantle. High source

temperatures, as inferred from high MgO contents, for the Belingwe komatiites are consistent with their derivation from a plume source. Their Nd isotope compositions ($\epsilon_{Nd} = 2.4$)¹ are consistent with the Belingwe komatiites having a recycled component in their source. Therefore, they are unlikely to be samples of the Archaean depleted mantle.

Results obtained for fossil glass inclusions support the suggestion that Archaean komatiites, in general, are ancient analogues of modern plume-related magmas^{2,3}. We are therefore in agreement with Kusky and Kidd¹² that the Belingwe greenstone belt may be a preserved oceanic plateau. There is no evidence for these komatiites being a sequence of convergent margin magmas¹³. □

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South America's earliest rodent and recognition of a new interval of mammalian evolution

André R. Wyss*, John J. Flynn†, Mark A. Norell‡, Carl C. Swisher III§, Reynaldo Charrier||, Michael J. Novacek‡ & Malcolm C. McKenna‡

* Department of Geological Sciences, University of California, Santa Barbara, California 93106, USA

† Department of Geology, Field Museum of Natural History, Roosevelt Road at Lake Shore Drive, Chicago, Illinois 60605, USA

‡ Department of Vertebrate Paleontology, American Museum of Natural History, Central Park West at 79th Street, New York, New York 10024, USA

§ Geochronology Center, Institute for Human Origins, 2453 Ridge Road, Berkeley, California 94709, USA

|| Departamento de Geología, Facultad de Ciencias Físicas y Matemáticas, Universidad de Chile, Casilla 13518, Correo 21, Santiago, Chile

THE mid-Cenozoic immigration of rodents and primates to South America (when it was widely isolated by oceans) represents a pre-eminent problem in the biogeographical history of placental mammals. The unexpected discovery of South America's earliest rodent in the central Chilean Andes provides information critical to resolving the source area and primitive morphology of South American caviomorphs, suggesting an African origin for the group. This rodent is part of a new fossil mammal fauna¹, the first diverse assemblage known for a critical 15–25 million year gap in the fossil record. We report here that co-occurrence of numerous higher-level taxa otherwise restricted to older or younger intervals identifies this fauna as representing a new biochronological interval preceding the Deseadan (South American Land Mammal Age), previously the earliest occurrence of rodents and primates on the

TABLE 1 Taxonomic list for the Tinguiririca fauna

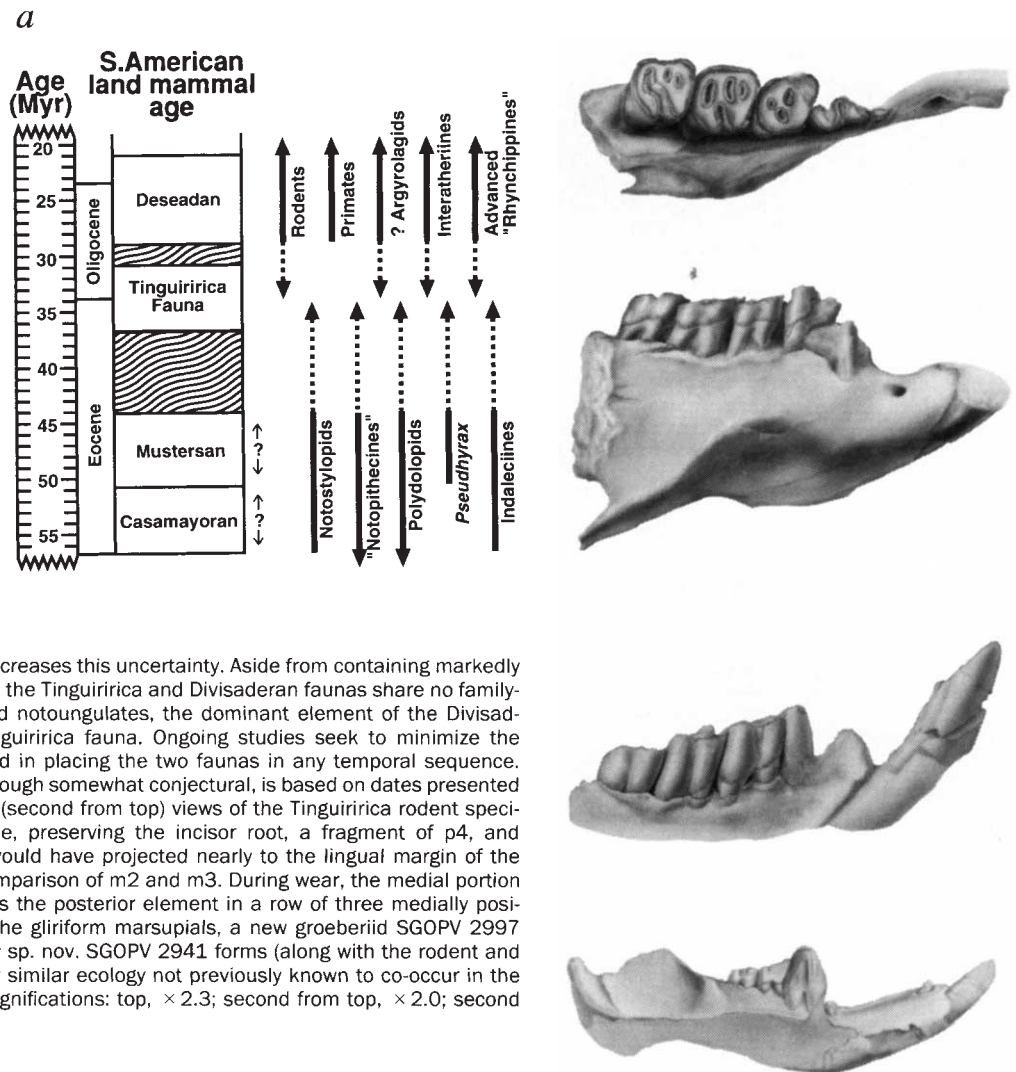
Marsupialia	Notoungulata
Groeberiidae	Notostylopidae
gen. et sp. nov.*	gen. et sp. nov.
?Argyrolagidae	Interatheriidae
gen. et sp. nov.	'Notopithecinae'
Polydolopidae	gen. et sp. nov.*
<i>Polydolops</i> , sp. nov.	Interatheriinae
Didelphimorphia inc. sed.	gen. et sp. nov. A
gen. et sp. nov.	gen. et sp. nov. B
Eutheria	Archaeohyracidae
Edentata	<i>Pseudhyrax</i> cf. <i>eutracytheroides</i>
?Dasypodidae inc. sed.	cf. ' <i>Bryanpattersonia sulcidens</i> '
Tardigrada	new taxon A
<i>Pseudoglyptodon</i> sp. nov.	new taxon B
Rodentia	new taxon C
?Dasyproctidae	new taxon D
gen. et sp. nov.	Homalodotheriidae
Litopterna	<i>Trigonolophodon</i> cf. <i>elegans</i>
?Adiantidae	Notohippidae
Indaleciinae	'Rhynchippinae'
gen. et sp. nov.*	<i>Eomorphippus</i> sp. nov.
Astrapotheria	' <i>Eomorphippus</i> ' cf. <i>pascuali</i>
(one taxon, unstudied)	new taxon A
	new taxon B
	Isotemnidae inc. sed.

Identifications are based on direct comparison with relevant collections in North and South American museums more fully detailed in a manuscript in preparation. 'Notopithecinae' and 'Rhynchippinae' are placed in quotes to reflect their almost certain paraphyly. Placement of '*Bryanpattersonia*' and '*Eomorphippus*' in quotes reflect nomenclatural problems detailed in work in progress. Asterisks indicate taxa that are either unknown in Patagonia or show closest affinities to low-latitude forms, including to a preliminarily described fauna from northwest Argentina¹⁹, and to the Casamayoran fauna from the Lumbra Formation also from northern Argentina²⁰.

continent. Radioisotopic dating corroborates biostratigraphy in identifying the new Andean rodent as the earliest known from the continent.

A diverse Palaeogene mammal fauna (>400 specimens) (Table

FIG. 1 a, Chronology of Eocene and Oligocene South American Land Mammal Ages, and biostratigraphic distribution of certain mammalian taxa in the Tinguiririca fauna. Dashed lines indicate range extensions, and striped blocks faunal hiatuses. Calibration of the Deseadan is based on ref. 7, data presented here, and work in progress; lack of radioisotopic tie points for the Mustersan and Casamayoran is signified by queries. The poorly known and undated Divisaderan Land Mammal Age is omitted for sake of clarity. Composition of the Tinguiririca fauna argues against recent^{7,27} or traditional suggestions that the Divisaderan is Deseadan, early Deseadan, or latest pre-Deseadan in age. Temporal placement of the Divisaderan relative to other better known Eocene-Oligocene SALMAs is not satisfactorily resolved; discovery of the Tinguiririca fauna only increases this uncertainty. Aside from containing markedly different groeberiids and indaleciines, the Tinguiririca and Divisaderan faunas share no family-level resemblances. Oldfieldthomasiid notoungulates, the dominant element of the Divisaderan fauna, are unknown in the Tinguiririca fauna. Ongoing studies seek to minimize the significant contradictions encountered in placing the two faunas in any temporal sequence. Duration of the Tinguiririca fauna, although somewhat conjectural, is based on dates presented in Table 2. b, Crown (top) and lateral (second from top) views of the Tinguiririca rodent specimen, SGOPV 2933, a right mandible, preserving the incisor root, a fragment of p4, and complete m1-3. A deep hypoflexid would have projected nearly to the lingual margin of the molars when unworn as shown by comparison of m2 and m3. During wear, the medial portion of the hypoflexid becomes isolated as the posterior element in a row of three medially positioned fossettids. Shown below are the gliriform marsupials, a new groeberiid SGOPV 2997 (second from bottom) and *Polydolops* sp. nov. SGOPV 2941 forms (along with the rodent and a possible argyrolagid) of presumably similar ecology not previously known to co-occur in the South American Cenozoic record. Magnifications: top, $\times 2.3$; second from top, $\times 2.0$; second from bottom, $\times 4.9$; bottom, $\times 1.0$.



1) is the result of five seasons of fieldwork in the Tinguiririca River valley, near Termas del Flaco, ($34^{\circ} 59' S$, $70^{\circ} 26' W$) central Chile. This Tinguiririca Fauna derives from volcanoclastic sediments previously attributed to the poorly dated ?late Cretaceous Colimapu Formation^{2,5}, but recently shown to pertain to the Abanico Formation⁶. The age of this latter unit, too, was previously poorly constrained, in part due to lack of informative fossils. Our discovery of fossil mammals and application of single crystal laser fusion $^{40}\text{Ar}/^{39}\text{Ar}$ dating has vastly improved the geochronology for this and related deposits, establishing a much younger age than previously thought for many of them.

In addition to its rodent, the Tinguiririca fauna contains a suite of endemic 'ungulates' known elsewhere only from Deseadan and younger deposits (for example, advanced 'rhynchippine' notohippids, several archaeohyracids closely related to *Archaeohyrax patagonicus*, and interatheriines). Similarly, the Tinguiririca fauna contains numerous taxa known elsewhere only from Mustersan and older horizons (such as a notostylopoid, indaleciine, notopithecine, polydolopid and *Pseudhyrax*). Co-occurrence of these formerly temporally disjunct taxa establishes the Tinguiririca fauna as a previously unrecognized pre-Deseadan, post-Mustersan (and non-Divisaderan) interval of South American land mammal evolution (Fig. 1a).

Multiple single crystal laser fusion $^{40}\text{Ar}/^{39}\text{Ar}$ dates constrain the Tinguiririca fauna (Table 2). Dates from immediately above the fossiliferous horizon indicate the fauna to be at least as

young as about 31.5 Myr (early Oligocene); dates from levels immediately below (but within the same stratigraphic unit as) the fossiliferous horizon indicate the fauna to extend potentially as old as late Eocene (~ 37.5 Myr). These represent the first dates available for the entire post-Riochican, pre-Deseadan span of the South American land mammal succession. As such, the Tinguiririca fauna supports the recently proposed late Oligocene age⁷ for the disputed lower temporal boundary of the Deseadan.

South America's earliest endemic rodent radiation (generally termed caviomorphs) involves forms with specialized mandibular (hystricognathous) and cranial (hystricomorphous) structure. The group's previously known earliest occurrences are in Deseadan localities in Argentine Patagonia and Bolivia^{8,10}. Because of their sudden diversity (some 15 species, in 7 families), and the previous pre-Deseadan faunal hiatus, caviomorphs are generally accepted as having reached South America earlier than the Deseadan, perhaps the only point of consensus regarding the group's origin.

Controversy stems in part from the group's initial diversity, which has led to debate about which of two major tooth crown patterns represents the primitive caviomorph condition. Conflicting interpretations are related to two radically different viewpoints about the phylogenetic affinities and biogeographical source area for the group. One hypothesis envisions five-crested upper molars as representing the ancestral caviomorph condition, implying affinities to dentally (and cranially) similar

TABLE 2 Tinguiririca fauna age

Analysis number	^{37}Ar	^{39}Ar	^{36}Ar	^{39}Ar	$^{40}\text{Ar}^*/^{39}\text{Ar}$	$\%^{40}\text{Ar}^*$	Age Myr $\pm$ s.d.
Sample 90CS-TDF20 (plagioclase)							
5259 01	5.6536	0.01943	53.0933	90.9	32.065	1.331	
5259 02	5.6216	0.02251	52.8218	89.4	31.902	0.704	
5259 03	3.3264	0.01558	50.2486	93.3	30.361	1.828	
5259 04	4.7746	0.00786	52.9391	96.4	31.972	0.504	
5259 05	5.1316	0.02409	51.3102	88.4	30.997	0.662	
5259 06	4.8035	0.08048	51.9572	68.9	31.384	1.220	
	Weighted mean =					31.646	0.320
altered grain?							
5259 07	23.0828	1.04709	91.2434	22.6	54.757	11.634	
Sample 90CS-TDF19 (plagioclase)							
5258 01	5.4000	0.00788	51.9069	96.4	31.354	0.468	
5258 02	6.8320	0.21235	51.9136	45.4	31.358	1.514	
5258 03	5.0410	0.03627	52.1694	83.4	31.511	0.539	
5258 04	5.7482	0.04452	51.6674	80.2	31.211	0.580	
5258 05	5.1570	0.01104	51.9845	94.7	31.401	0.365	
5258 06	5.0637	0.01760	52.6603	91.6	31.805	0.665	
5258 07	5.0960	0.00474	51.5102	98.1	31.117	0.446	
5258 08	4.5901	0.01085	51.7061	94.7	31.234	0.358	
	Weighted mean =					31.335	0.171
Combined weighted mean of TDF19 and 20 =					31.404	0.151	
Sample 90CS-TWH0 (plagioclase)							
5241 02	1.9288	0.12751	61.2378	62.0	37.307	1.154	
5241 05	0.3615	0.05299	60.9994	79.6	37.163	0.776	
5241 06	0.4021	0.15814	61.8658	57.0	37.685	1.078	
5241 07	0.4862	0.02445	62.0831	89.6	37.816	0.521	
5241 08	0.3676	0.00909	62.0903	95.9	37.820	0.512	
	Weighted mean =					37.671	0.305
contaminant grains?							
5241 01	0.4813	0.16370	64.9995	57.3	39.573	1.911	
5241-03	0.3814	0.03255	62.6420	86.7	38.153	0.896	
Sample 90CS-TWH0 (biotite)							
5242 01	0.0276	0.50394	61.3023	29.2	37.345	3.252	
5242 03	0.0617	0.44815	60.8075	31.5	37.047	2.572	
5242 04	0.0078	0.51079	61.2037	28.8	37.286	3.233	
5242 05	0.0433	0.38783	61.6036	35.0	37.527	2.357	
5242 06	0.0219	0.30230	60.9613	40.6	37.140	1.205	
5242 07	0.1022	0.39658	61.1127	34.3	37.231	2.372	
	Weighted mean =					37.216	0.849
altered grain?							
5242 02	0.0232	0.49516	51.5898	26.1	31.480	3.973	

$$\lambda_{\text{e}} + \lambda_{\text{e}'} = 0.581 \times 10^{-10} \text{ yr}^{-1}; \quad \lambda_{\beta} = 4.962 \times 10^{-10} \text{ yr}^{-1}; \quad {}^{40}\text{K}/{}^{40}\text{K}_{\text{total}} = 1.167 \times 10^{-4}$$

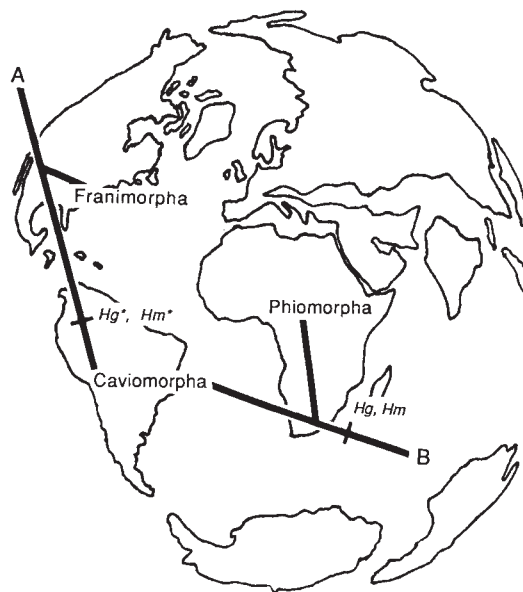
The age of the Tinguiririca fauna was ascertained through $^{40}\text{Ar}/^{39}\text{Ar}$ laser total-fusion techniques applied to plagioclase and biotite separated from volcanoclastic horizons of the Abanico Formation. Mineral grains were treated in an ultrasonic cleaner with dilute (~0.7%) hydrofluoric acid for 5 min to remove any altered glass and adhering clays, then rinsed for 5 min in distilled water. They were irradiated together with a centrally located monitor mineral (Fish Canyon Tuff sanidine), for 30 min in the hydraulic rabbit core facility of the Omega West research reactor at Los Alamos National Laboratory following previously described procedures. After irradiation, single crystals of the monitor mineral and Abanico samples were loaded into individual 2 mm diameter wells of a copper sample disk then placed within the sample chamber of the extraction system, and baked out at 200 °C for 8 h. Total fusion of the samples and monitor mineral was accomplished with a 6W Coherent Ar ion laser. Released gases were purified by two Zr-Fe-V getters operated at 150 °C, and condensable gases were collected on a cold-trap operated at -45 °C. Argon was measured in an on-line Mass Analyzer Product 215 noble-gas mass spectrometer operated in the static mode. Laser heating, gas purification, and mass spectrometry were completely automated following computer programmed schedules²¹. The $^{40}\text{Ar}/^{39}\text{Ar}$ ages were calculated using a *J* value determined from replicate analyses of individual grains of the co-irradiated monitor mineral Fish Canyon Tuff sanidine (FC) with a reference age of 27.84 Myr intercalibrated in-house with Minnesota hornblende MMhb-1 with a published age of 520.4 Myr^{22,23}. The $^{40}\text{Ar}/^{39}\text{Ar}$ dating of single crystals of plagioclase and biotite separated from sample 90CS-TWH0, the stratigraphically lowest sample dated from the Abanico Formation, yielded weighted mean ages of 37.67 ± 0.31 Myr (s.e.) and 37.22 ± 0.85 Myr (s.e.), respectively. Samples 90CS-TDF20 and 90CS-TDF19 yielded concordant ages of 31.65 ± 0.32 Myr (s.e.)²⁴ and 31.34 ± 0.17 Myr (s.e.). A combined weighted mean of both of these samples indicate an age of 31.40 ± 0.15 Myr (s.e.). The maximum output of the laser during this study was ~8 watts. Ca and K corrections were determined from laboratory salts: $(^{36}\text{Ar}/^{37}\text{Ar})\text{Ca} = 2.6 \times 10^{-4} \pm 5.0 \times 10^{-5}$, $(^{39}\text{Ar}/^{37}\text{Ar})\text{Ca} = 6.7 \times 10^{-4} \pm 3.0 \times 10^{-5}$ and $(^{40}\text{Ar}/^{39}\text{Ar})\text{K} = 2.03 \times 10^{-2} \pm 4.0 \times 10^{-4}$. *J*, the irradiation parameter, is based on replicate single-crystal analyses of the monitor mineral Fish Canyon Sanidine and is 0.0003412 ± 0.0000005 for sample 90CS-TWH0 and 0.000338 ± 0.0000005 . Mass discrimination during this study, as determined by replicate air aliquots delivered from an on-line pipette system, was 1.006 ± 0.0002 . Decay constants are those recommended by refs^{25,26}.

* Radiogenic.

African phiomorphs⁹. Alternatively, four-crested upper molars have been argued to be primitive for the Caviomorpha, a view consistent with the suggested derivation of caviomorphs from cranially conservative, North American franimorphs^{8,10}. As the earliest South American rodent known, the Tinguiririca form offers a test of these alternatives.

BOX 1 Competing hypotheses

SUMMARY of two major competing hypotheses of caviomorph phylogenetic and biogeographical affinities (late-Eocene, Lambert equal-area projection²⁸). Hypothesis A, long advocated by Wood^{8,10}, proposes North America as the source area for the Caviomorpha. This hypothesis, usually not expressed in explicit phylogenetic systematic terms, envisions derivation of caviomorphs from early Eocene franimorph paramyids (doubtless a paraphyletic assemblage), in general, and reithroparamyines in particular. These presumptive North American 'ancestors' are plesiomorphic in most cranial and mandibular features. For example, they are characterized by a skull structure (protrogomorphy, an unenlarged infraorbital foramen and correspondingly conservative pattern of jaw musculature) which is primitive for the Rodentia and unlike the advanced condition typical of caviomorphs (hystricomorphy, an enlarged infraorbital foramen for passage of a derived pattern of jaw musculature). In the configuration of the lower jaw, these North American forms are at best only incipiently developed toward the advanced condition characterizing caviomorphs (hystricognathy), or at worst indistinguishable from the condition primitive for rodents^{29,30}. Acceptance of Hypothesis A



requires the convergent development (indicated by asterisk) of hystricognathy and hystricomorphy (Hg and Hm) in caviomorphs and a group of Old World forms (phiomorphs⁹) also typified by these advanced features, as well as numerous additional parallel developments. Hypothesis B, advocated by Lavocat⁹, proposes an African origin for the Caviomorpha, with the presence of hystricognathy and hystricomorphy in New and Old World forms attributed to inheritance from a common ancestor. Information provided by the new mandible from the Andes of central Chile, the oldest rodent known from the continent, argues in favour of hypothesis B, as does other recent morphological evidence³¹; the case for a North American franimorph origin for caviomorphs, though not impossible, is rendered that much more unparsimonious, however. Outstanding remaining questions include identification of which Old World hystricognath group (given that Phiomorpha is probably paraphyletic) is most closely related to the caviomorphs, and whether, in fact, caviomorphs are monophyletic with respect to all known Old World hystricognaths. These details aside, there is no morphological basis for the recent suggestion³² (based on amino-acid sequence data) that the guinea pig (*Cavia porcellus*, a caviomorph and the only hystricognath included in that study) is not a rodent. □

The Tinguiririca rodent (Fig. 1b) most resembles *Branisamys* from Bolivia but is ~20% smaller; otherwise it is not closely comparable to described Deseadan forms. The dentition is worn, but moderately high crowns (for an early caviomorph) allow considerable inferences about its original morphology (Fig. 1b). Of importance are three medially positioned fossettids on the molars. Among known Deseadan rodents, lower molars producing this pattern during wear (*Branisamys* and *Protosteiromys*) are associated with a five-crested upper molar pattern. The Tinguiririca rodent thus almost certainly possessed a similar five-crested upper molar pattern. This, coupled with the observation that erethizontids (New World porcupines), which are usually considered the sister group of the remaining caviomorphs (based on diverse anatomical^{11, 13}, karyological¹⁴, and some molecular¹⁵ evidence), also possess this pattern, strongly suggests that the five-crested upper molar pattern represents the ancestral condition for the Caviomorpha. This in turn supports a close phylogenetic relationship between New and Old World hystricognaths, and Africa as the most likely source area for caviomorphs.

The Tinguiririca fauna has several important palaeoecological and palaeoclimatic ramifications. It documents the coexistence of at least three higher-level taxa with an enlarged anterior (gliriform) tooth (Fig. 1b), including both marsupials (groeberiids, polydolopid and possible argyrolagid) and a rodent, an unprecedented association of such presumably ecologically similar forms. A striking feature of the Tinguiririca fauna is the absence of most low-crowned herbivorous taxa which dominate pre-Deseadan faunas elsewhere in South America¹⁶. The Tinguiririca fauna represents the earliest South American mammalian community dominated by high-crowned (hypsodont) herbivores, the appearance of which is probably related to climatic shifts, the establishment of seasonally drier, more open habitats, and more abrasive diets^{17, 18}. Moreover, the degree of hypsodonty seen in the Tinguiririca herbivore fauna was achieved significantly earlier in South America than in various lineages on the northern continents¹⁸. With discovery of the Tinguiririca fauna, the base of Simpson's "Second Faunal Stratum" (previously equated with the Late Oligocene⁷ base of the Deseadan) is pushed to near (or perhaps below) the Eocene–Oligocene boundary. And last, the Tinguiririca fauna exemplifies a comingling of Patagonian and more northern South American faunistic regions stemming from early Cenozoic latitudinal zonation¹⁶.

Equally dramatic Andean geotectonic implications of the Tinguiririca fauna include new documentation of several regional unconformities and extensive thrusting, and a shift in age (perhaps 50 Myr younger) of a major pulse of central Andean volcanism.

Finally, if as is often assumed, rodents and primates arrived in South America roughly simultaneously, these hiatus-filling Andean deposits afford an unparalleled opportunity for discovery of platyrrhine primates significantly older than those presently known. □

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Telling tails explain the discrepancy in sexual partner reports

M. Morris

Isaac Newton Institute for Mathematical Sciences,
University of Cambridge, 20 Clarkson Road,
Cambridge CB3 0EH, UK
Department of Sociology, Columbia University, New York,
New York 10027, USA

AN anomaly often noted in surveys of sexual behaviour is that the number of female sexual partners reported by men exceeds the number of male partners reported by women^{1–3}. This discrepancy is sometimes interpreted as evidence that surveys produce unreliable data due to sex-linked response and sampling bias. We report here that among the 90% of respondents reporting fewer than 20 lifetime partners, however, the ratio of male to female reports drops from 3.2:1 to 1.2:1. The anomaly thus appears to be driven by the upper tail of the contact distribution, an example of the general principle of outlier influence in data analysis. The implication is that sexual behaviour surveys provide reliable data in the main, and that simple improvements can increase precision in the upper tail to make these data more useful for modelling the spread of AIDS and other sexually transmitted diseases.

An example of the reporting anomaly is given in Table 1. In these data, men report well over three times as many lifetime partnerships with women as women report with men. This creates a massive discrepancy, with more than 60% ((20,523 – 6,371)/22,105 × 100 = 64.0%) of all contacts reported by men unaccounted for. The many explanations suggested for this discrepancy^{4–6} stem from two basic issues: sample bias and reporting bias.

Sample bias can explain the observed ratio if important parts of the population are systematically excluded. If men are typically older than their female partners, men in the sample may be reporting contacts with women who fall below the sampled age range. To account for the discrepancy, however, more than 60% of the lifetime sexual partners reported by all men would have to be women who are currently under 18 years old, a highly unlikely scenario. The exclusion of prostitutes (commercial sex workers, or CSWs) from the sample is another potential sample bias. The General Social Survey (GSS) and other survey data suggest that the fraction of men who visit CSWs is of the order of 3% over a 5-year period². This is likely to be an underestimate,

but even if half of the men in these data were reporting unattributed CSW contacts, each man would have to have an average of about 15 different CSW partners. Alternatively, it would require about 10–20 CSWs (at 1,000–2,000 lifetime partners) to compensate for the discrepancy. CSWs would then comprise 0.5–1.0% of the female population, a substantially higher fraction than estimated⁷. Sex-linked travel biases could also affect the observed ratio, but travel-related sexual contacts are unlikely to comprise more than half of all male contacts. Reporting bias could explain this ratio if under- and over-reporting patterns are sex-linked, but one would have to assume something like all men over-reporting by 65% and all women simultaneously under-reporting by more than 200%.

Recent commentaries have suggested that the bias is more specifically located in the upper tail of the reported partnership distribution^{4,5}, and this explanation is supported by the GSS data. About 90% of the sample report fewer than 20 partners in their lifetime (see Fig. 1a. for the full contact distribution), and for this group the report ratio is close to 1 (Table 2a). This qualitative pattern is reproduced in every year of the GSS data, for both lifetime and current-year partnerships (Table 2b). The point driven home by Tables 1 and 2 is that explanations for the male:female report discrepancy should be focused on the upper tail of the contact distribution.

Two important implications can be drawn from these findings. The first concerns a substantive explanation for the remaining discrepancy in the tail, and the second concerns modelling of sexually transmitted diseases (STDs).

One way of accounting for the anomalous tail behaviour is suggested by the uniformly lower discrepancies found in current-year reports in Table 2b. Shorter time frames are probably less prone to reporting error than lifetime totals. Some evidence for this is that more than 75% of the upper tail respondents report lifetime totals in round numbers (Fig. 1a). Rounding indicates uncertainty, and in these circumstances gender norms are likely to influence the direction of the rounding and the observed discrepancy.

Simple improvements in data collection can improve precision here. Shorter reporting time frames will reduce both errors of recall and the amount of non-disclosure, as smaller totals are less likely to provoke intentional under-reporting. Supplemental interview schedules could also be used for very active respondents, with reporting time frames either event-based, for example, "when were the dates of your five most recent contacts", or constant but short, for example a week. If estimates of lifetime totals are desired, then either life-cycle data collection methods or synthetic cohort analysis should be adopted⁸.

The second issue concerns the use of contact data for mod-

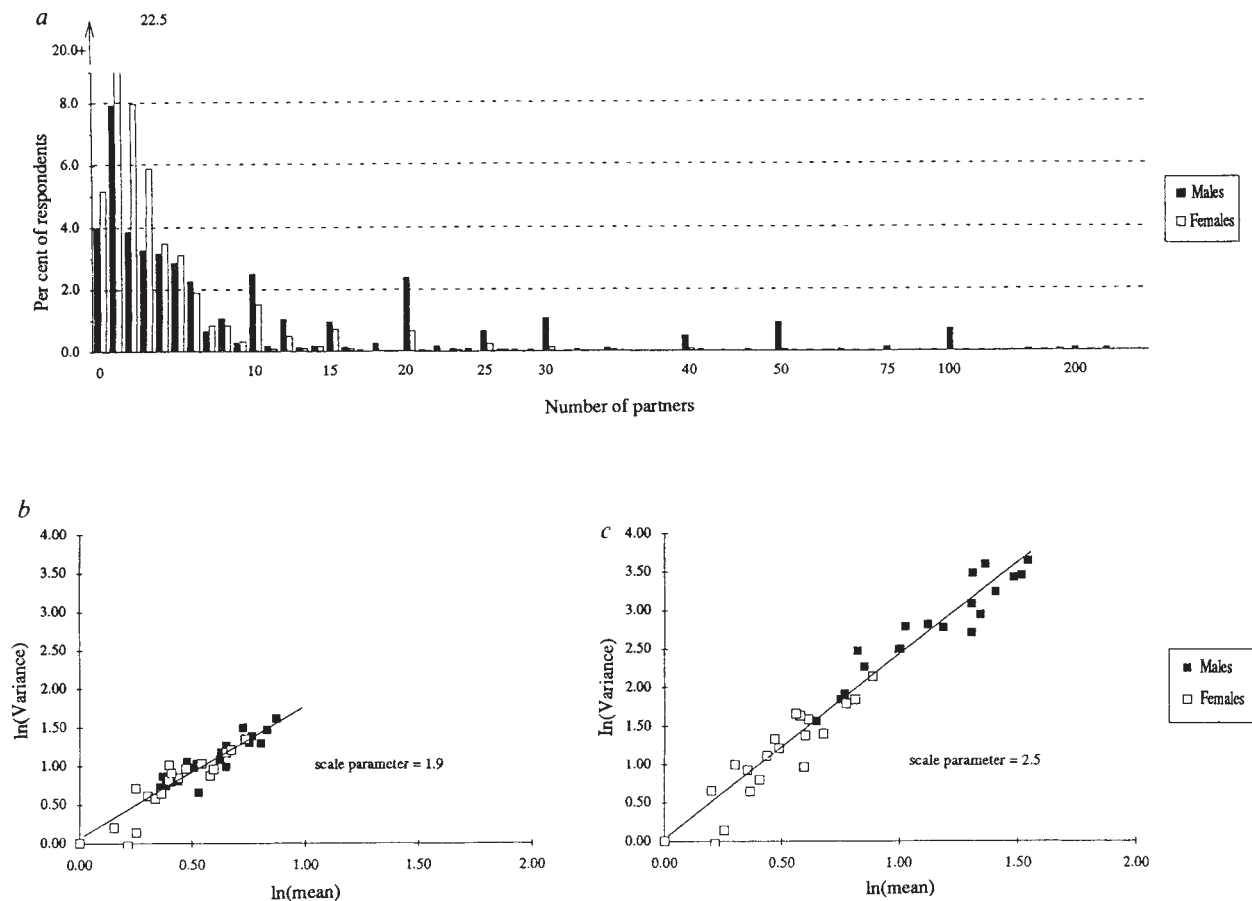


FIG. 1 Plots of the contact frequency distribution and variance to mean scaling. a, The frequency distribution of lifetime contacts for men and women in the GSS sample. The 'heaping' of reports on round numbers in the upper tail is quite pronounced. Note that there are consistently more men reporting in the upper tail. b, c, The logged (base e) variance to mean plots for the full sample (c) and the sample restricted to respondents with fewer than 20 lifetime partners (b), showing the effect of the tail on the estimated scaling parameter. The observations represent 40 different demographic subgroups formed by a cross tabulation of race (white, non-white), sex, age (5 categories) and marital status (married, other). Male and female groups are identified by the plotting

characters. The scale parameter is the estimated coefficient obtained from a weighted regression through the origin of the two logged variables. Previous studies found a scaling exponent of 3.2 using comparative data on heterosexual and homosexual contact patterns¹³. The lower value of 2.5 found in the full sample here is probably attributable to the relative homogeneity of the GSS respondents. The effect of tail behaviour is still clear, however, as restricting the sample to less active 90% both lowers the estimated scale parameter and substantially reduces the male-female distinction in the plot. This appears to contradict the theoretical prediction that populations with lower mean contact rates should generate exponent values around 4 (ref. 14).

elling and controlling the population dynamics of STDs. The role of 'core groups' in the upper tail of the contact distribution is critical and can drive an STD epidemic^{9,11}.

One common way of incorporating core groups into epidemic simulation studies is to increment the mean contact rate (μ) by the coefficient of variation (σ^2) (ref. 12). A power function of the form $\sigma^2 = a\mu^b$ (where a and b are constants) fits observed data remarkably well¹³, and some explanations and implications have been suggested^{14,15}. In panels *b* and *c* of Fig. 1, the means and variances of 40 demographic subgroups in the GSS are plotted, for the full sample and for those reporting less than 20 lifetime partners. The effect of tail behaviour is strong: restricting the sample to less active respondents reduces both the male-female distinction in the plot and the size of the estimated power exponent. Given the level of uncertainty and error in upper-tail reports, scale estimates derived from the raw coefficient of variation should be used with caution.

Another method for investigating core group effects is through simulation of disease dynamics in populations divided into activity classes. To adjust for the male-female discrepancy, explicit assumptions have to be made regarding both reporting error and the mixing patterns among activity classes. This can best be appreciated with a simple example, taking the male contact rates as correct and adjusting female reports to match (Fig. 2). If random or proportional mixing is assumed then the excess male contacts will be spread across both low- and high-activity women, implying a general under-reporting bias for both. If instead all mixing is assumed to be within activity class (perfect assortative mating) and excess contacts are due to the more active males, then these contacts would be distributed only to

TABLE 1 Total lifetime number of partners reported in each sex category

Respondent:	Partner:		Total partners	Total respondents*	M:F report ratio
	Males	Females			
Males	1,582	20,523	22,105	1,691	3.22
Females	6,371	241	6,612	1,845	
				3,536	

The values in the cells of this table represent the total number of sexual partners reported by all respondents in the row category with persons in the column category. Respondents were asked to report the number of male and female sexual partners they had had since they were 18 years old (referred to here as 'lifetime' partners). In theory, if the number of males and females is representative of the population sex ratio, the table should be symmetric: the number of female partners reported by men (upper right cell) should equal the number of male partners reported by women (lower left cell). The sample, instrument and field methods used to collect the data in Table 1 are the highest quality available. The GSS is an annual cross-sectional survey, using face-to-face interviews and a stratified probability sample designed to be representative of the US population 18 years old and older¹⁹. The survey has been done every year for the past 20 years, and response rates are 71–80%, among the highest in the field. The questions on sexual behaviour were added in 1989, designed to provide a small amount of reliable representative data on current patterns of adult sexual behaviour in the general population when another more comprehensive survey was embroiled in funding controversy. These items are distributed as a self-administered questionnaire to minimize the potential discomfort and embarrassment of the respondent. Non-response is less than 10% overall, and less than 5% for those under 55 years old.

* Analyses are based on the data weighted to match the 1990 census age and sex profile.

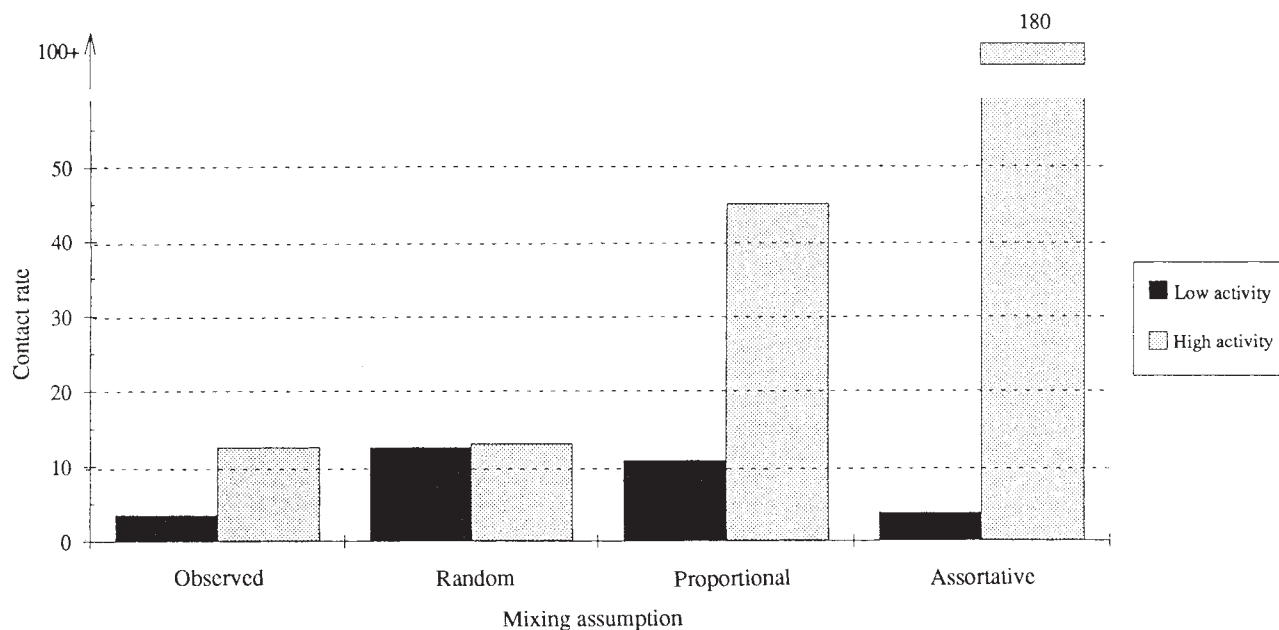


FIG. 2 Female contact rates for different mixing assumptions. Alternative ways of distributing the excess contacts in this data set to low- and high-activity women are examined here. Observed contact rates are shown in the left-hand bars. Assumptions about the mixing between high- and low-activity classes strongly affects the distribution of the excess male contacts and the implied increases in the contact rates for each class of women. For the less active women, implied contact rates under random and proportional mixing are over three times higher than observed rates. For the more active women random mixing nearly

reproduces the observed rate but proportional mixing raises it by 400%. If all mixing is assumed to be within activity class and the excess contacts are due to the more active males, then these contacts would be distributed only to the more active women. This leaves the observed contact rates for the less active women unchanged, but requires either massive increases in the contact rates for the more active women, or the assumption of a number of very high-activity women excluded from the sample. The different assumptions strongly affect the resulting disease dynamics.

TABLE 2 Partner tables broken down by contact rate

(a) Male:female ratio for lifetime total partners and full data set

Respondent:	Partner:		Total partners	Total respondents	Sample fraction	M:F report ratio
	Males	Females				
< 20 Partners						
Males	192	5,852	6,044	1,363		
Females	4,921	158	5,079	1,792		
				3,155	89.23	1.19
20+ Partners						
Males	1,390	14,671	16,061	328		
Females	1,450	83	1,533	53		
				381	10.77	10.12

(b) Male:female report ratios for different time frames, cutpoints and years

Time frame	Cutpoints	Sample fraction	All years	Male:female report ratio based on:		
				1989	1990	1991
Lifetime	All	100.0	3.22	3.43	3.23	3.01
	< 20 partners	89.2	1.19	1.33	1.17	1.07
	< 30 partners	94.2	1.58	1.75	1.63	1.38
	< 50 partners	96.9	1.87	1.99	2.07	1.57
	20+ partners	10.8	10.12	10.74	10.50	9.27
Current year	All	100.0	1.81	1.50	2.22	1.66
	< 5 partners	97.0	1.12	1.15	1.22	1.01
	5+ partners*	3.0	8.57	4.55	11.05	-
	Unadjusted sample N		3,368	1,322	1,091	1,225

The effects of upper tail of the contact distribution on the M:F report ratio. a, For the 90% of respondents reporting less than 20 lifetime partners, male and female reports are substantially closer. There is still a slightly larger number of partnerships reported by men, but the discrepancy has fallen by nearly an order of magnitude: from over 200% to just under 20%. Among respondents in the upper 10% tail of the contact distribution, by contrast, men report over 10 times more partnerships than women. b, the same pattern of tail extremity in report ratios is reproduced for each year in the GSS data and for both the reported lifetime number of partners and the number of partners reported in the current year. Male reports only slightly exceed female reports for most of the population (90–98% of the sample, depending on the time frame and cutpoint chosen), but dramatically exceed them in the upper tail. The numbers in the upper tail are quite small for the individual years, so the ratio is much more variable than for the combined total over all years. No women reported 5+ male partners in 1991, so the ratio cannot be calculated.

* The reporting categories for number of sexual partners in the current year are exact for zero to four partners, but are specified as ranges for five or more partners: 5–10, 10–20, 20–100 and over 100. The ratios were calculated assuming the midpoints of these intervals (7.5, 15, 60 and 200). There is no difference in qualitative results, and little difference in the actual values of the report ratio if either the lower or upper bound are instead assumed.

the more active women. The mixing and reporting error assumptions are linked, and they can dramatically change the simulated effects of core groups. In general, the greater the mixing, the greater the effect of core groups on the general population, but even this general rule depends on the stability of core group membership.

To address these problems, qualitatively different data are needed: mixing patterns and life-cycle changes. Mixing patterns are largely defined by simple demographic attributes, and for such attributes partner data are relatively easy to obtain from respondent reports. Standard survey sampling methods for 'ego-centric network data' can therefore be used¹⁶. These methods are less intrusive than snowball sampling or contact tracing, and are likely to provide sufficient detail for most modelling and surveillance purposes^{17,18}. Life-cycle patterns of sexual behaviour also need to be analysed if the term 'core group' is to have real analytical coherence. People clearly move into and out of core

group behaviour over their lifetime, triggered by changes in residence, marital status and short-term travel, and these changes have strong implications for core group effects on disease spread. Life-cycle data collection strategies can be used to track these patterns.

The analysis here demonstrates that sexual behaviour surveys can produce reliable measures of sexual conduct for the great majority (90–97%) of survey respondents. Simple improvements in the data collection methods could substantially enhance the usefulness and precision of the data: shorter retrospective time frames for more active respondents and the collection of some life-cycle and network information. These improvements will increase reliability in measures of the upper tail of the contact distribution, and make it possible to identify core groups accurately. The role of behaviour and behavioural change in the spread of STDs can then be systematically assessed. □

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Dopamine D4 receptors elevated in schizophrenia

Philip Seeman^{*†}, Hong-Chang Guan^{*}
& Hubert H. M. Van Tol^{*†‡}

^{*} Pharmacology and [†] Psychiatry Departments,
Medical Sciences Building, University of Toronto, Toronto,
Ontario, Canada M5S 1A8

[‡] Laboratory for Molecular Neurobiology, Clarke Institute of Psychiatry,
250 College Street, Toronto, Ontario, Canada M5T 1R8

ALTHOUGH the biological basis of schizophrenia is not known, possible causes include genetic defects, viruses¹, amines², brain structure and metabolism^{3–5}, neuroreceptors^{6–8}, and G proteins⁹. The hypothesis of dopamine overactivity in schizophrenia is based on the fact that neuroleptics block dopamine D2 receptors in direct relation to their clinical antipsychotic potencies^{10,11}. Moreover, dopamine D2 or D2-like receptors are elevated in postmortem schizophrenia brain tissue^{8,12,13}. This elevation, however, is only found *in vivo* using [¹¹C]methylspiperone¹⁴ but not [¹¹C]raclopride¹⁵. The dopamine D4 receptor gene¹⁶ has not yet been excluded in schizophrenia because the 21 gene variants¹⁷ of D4 have not yet been tested. Because the link between D1 and D2 receptors is reduced in schizophrenia tissue⁹, we tested whether one component of this link was sensitive to guanine nucleotide. We report here that the binding of [³H]raclopride to D2 receptors in schizophrenia was not sensitive to guanine nucleotide. This finding permitted analysis of data^{12,18} on the binding of [³H]lemonapride to the D2, D3 and D4 receptors. We conclude that the combined density of D2 and D3 receptors (labelled by [³H]raclopride^{16,19}) is increased by only 10% in schizophrenia brain, as found by Farde *et al.*¹⁵, but that it is the density of dopamine D4 receptors which is sixfold elevated in schizophrenia. These findings resolve the apparent discrepancy, mentioned above, wherein the density of [¹¹C]methylspiperone-labelled sites¹⁴ (D2, D3 and D4), but not that of [¹¹C]raclopride-labelled sites¹⁵ (D2 and D3), was found elevated in the schizophrenia striatum.

The effect of guanine nucleotide on the density of dopamine D2 receptors was tested using [³H]raclopride and a centrifugation method^{9,20} (Fig. 1 legend). The density of [³H]raclopride sites was increased by 21.6 ± 3.7% (5 control brains, 5 Alzheimer brains, and 11 Huntington brains), when the brain tissue was incubated with guanine nucleotide (Table 1). This compares to an increase of only 1 ± 0.3% for 17 schizophrenia brains (Table 1; Fig. 1). One Huntington brain tissue was in the schizophrenia range. Two washes of the homogenates yielded absolute densities about 25% lower than the unwashed tissues (Fig. 1 legend) but the rise in the binding of [³H]raclopride caused by the guanine nucleotide was still about 20% in five control tissues. This nucleotide-induced elevation of [³H]raclopride binding in twice-washed tissues is the same as that reported by Lazareno²², who found that GTP enhanced the binding of the D2 antagonist [³H]domperidone in membranes that had been washed up to four times. Moreover, guanylimidodiphosphate (200 µM) elevates the density of [³H]spiperone binding sites to dopamine D2 receptors by 25% in anterior pituitary tissue²³, where little or no endogenous dopamine exists, and by about 10% in cloned D4.2 and D4.7 dopamine receptors (H.H.M.V.T. and P.S., unpublished data) in the absence of dopamine. (The competition between dopamine and [³H]raclopride was also tested on these tissues to measure the proportion of agonist high-affinity sites; however, the proportion of these sites in control tissues at 2 nM [³H]raclopride was only about 8 ± 5%, precluding any reliable measurements, given the limited amount of human tissue from a single brain. Furthermore, to determine whether guanylimidodiphosphate altered the dissociation of dopamine from the D2 receptor, we attempted to measure the dissociation kinetics of [³H]dopamine in the control and schizophrenia tissues. Such

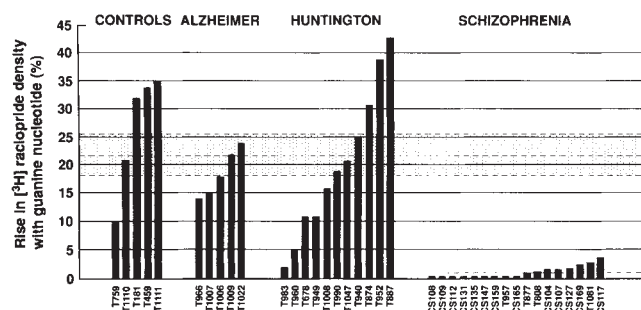


FIG. 1 The density of [³H]raclopride binding sites in striata from control, Alzheimer, and Huntington brain was elevated 21.6% by incubation with 200 µM guanylimidodiphosphate. In schizophrenia brain, the elevation averaged only 1%. Average values for 2–4 experiments.

METHODS. Human frozen striata were blotted, weighed frozen, and buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM KCl, 1.5 mM CaCl₂, 4 mM MgCl₂, 120 mM NaCl) added to yield 4 mg tissue per ml. The suspension was homogenized (glass-Teflon; ten up-down strokes) but not washed, because our previous work²¹ had shown that the customary two washes of homogenized tissues resulted in a loss of 30–60% of dopamine receptors in human tissues. Polypropylene microfuge tubes (1.8 ml) received 0.25 ml buffer, 0.25 ml of [³H]raclopride (12 final concentrations, from 0.2 to 20 nM), and 0.5 ml of the tissue homogenate which had been pre-incubated for 1 h in either buffer alone or in buffer containing 400 µM guanine nucleotide (guanylimidodiphosphate; Sigma), the final concentration being 200 µM. After 2 h at 20° the tubes were centrifuged in the horizontal position at 11,000g for 6 min in a Beckman 12 microfuge at room temperature. The supernatants were aspirated. The pellet-containing tip of each tube was cut off and put into a scintillation minivial. The minivials received 4 ml each of scintillant and were monitored 6 h later for tritium in a Packard 4660 scintillation spectrometer at 55% efficiency. Nonspecific binding was defined as that in the presence of 10 µM S-sulpiride (Ravizza, Milan). The density of [³H]raclopride binding sites (B_{max}) and the dissociation constant, K_D , were obtained by Scatchard analysis. When the K_D was higher than 10 nM, the results were unreliable, with considerable scatter; such data occurred in 5 tissues and these results were not included. Some of the tissues were also tested with [³H]ketanserin (72 Ci mmol⁻¹) to measure serotonin S2 and 1C receptors, and with [³H]SCH 23390 (60 Ci mmol⁻¹) to measure dopamine D1 receptors; the methods were identical to that used for [³H]raclopride, except the final concentrations were 0.05 to 5 nM, and nonspecific binding was defined by 1 µM (+)butaclamol¹².

results, however, were inconclusive, presumably because [³H]dopamine has a 10-fold higher affinity for the dopamine D1 receptor than the D2 receptor²⁴. Our data with the D2-selective agonist, [³H]quinpirole²⁵, were also inconclusive, presumably as a result of this ligand having a high dissociation constant of 6.8 nM²⁵. We plan to develop and test [³H](+)-PHNO ((+)-4-propyl-9-hydroxy-2,3,4a,5,6,10b-hexahydro-naphth[1,2b][1,4]oxazine) to label the agonist state of the D2 receptor in these tissues with and without guanine nucleotide, because (+)PHNO is selective for D2 receptors with a low dissociation constant of about 0.5 nM²⁴.)

The density of [³H]ketanserin sites (for serotonin S2 and S1C receptors) was not influenced by guanine nucleotide in either control or schizophrenia tissues, consistent with other findings²⁶; the density was 2.2 ± 0.3 pmol g⁻¹ in control tissues and 2.8 ± 0.7 pmol g⁻¹ in schizophrenia tissues. Guanine nucleotide elevated the density of [³H]SCH 23390 sites (dopamine D1 and D5 receptors) by 29 ± 8% ($N=5$) in control tissues, but lowered the density by 19.2 ± 3% in schizophrenia tissues ($N=6$); the densities were similar to those previously reported¹².

The insensitivity of [³H]raclopride binding to guanine nucleotide in the schizophrenia tissues could not be attributed to the age of the patients, because the average age of the schizophrenia patients was 64.9 ± 4 years, the same as the average age of 64.2 ± 4 years for all the other brains tested (Table 1). Moreover,

TABLE 1 Record of illness and drug treatment, and effect of guanine nucleotide on [³H]raclopride density

Controls	Age (yrs) sex	PM-delay (h)	Cause of death	Main drug (years)	Years ill	Main sign, symptom or organ	Region studied	[³ H]Raclopride		% rise
								B[K] (pmol g ⁻¹) [nM]	+Gpp[NH]p B[K] (pmol g ⁻¹) [nM]	
T 759	77m	13	cancer		~1	stomach	P	11.0[1.3]	12.1[1.1]	10%
T 1110	74f	11	CHF	chloram.	13	lymphoma	P	8.2[3.2]	9.9[5.8]	21%
T 181	87f	3		flurazepam			CN	7.9[1.5]	10.3[1.4]	32%
T 459*	85m	8	cancer	H(1)	~1	pneum.	P	28.4[6.8]	38.0[8.6]	34%
T 1111	68m	10	MI	none	0.5	CVA signs	P	8.4[3.1]	11.3[3.6]	35%
T 308*	27m		accid.	none	0	shock	P			
T 331*	61m	21	MI	oxycodone		alcoholism	P			
T 362*	73m	8	MI	TP, dig.	~1	angina	P			
Mean ± s.e.	69 ± 7	10.7 ± 2						12.8 ± 3.9[3.2]	16.3 ± 5[4.1]	26% ± 4.8%
Alzheimer's disease										
T 966	82m	11	CVA	Th, H	8	memory	P	18.6[6.0]	21.1[5.0]	14%
T 1007	70f	6	CVA	H (~3)	10	memory	P	13.6[1.5]	15.6[1.4]	15%
T 1006	84m	15	pulm.	L(1), c(8)	8	memory	P	21.2[1.2]	25.0[0.9]	18%
T 1009	79f	15		L	~7	memory	P	24.5[3.2]	27.2[2.5]	22%
T 1022	79m	16	CVA	dipyr. id.	2	confusion	P	14.4[1.8]	17.6[1.6]	24%
Mean ± s.e.	79 ± 2	12.6 ± 1.9						18.5 ± 2.1[2.7]	21.3 ± 2.2[2.3]	18.6% ± 1.9%
Huntington's disease										
T 983	64m	11	pneum.	H	~8	chorea	P	10.5[7.3]	10.7[7.0]	2%
T 960	58f	21	pneum.	H, c	13	paranoia	CN	13.8[4.6]	14.5[3.8]	5%
T 678	43m	3	pneum.	H	12	paranoia	P	14.3[1.4]	15.9[0.9]	11%
T 949	56m	6	pneum.	lorazepam	25	paralysis	P	1.8[2.1]	2.0[1.9]	11%
T 1008	46f	6	CVA	captopril	0	hyperten.	P	20.3[0.9]	23.5[1.0]	16%
T 990	78m	5	pneum.	diazepam	20	chorea	P	5.7[1.7]	6.8[2.7]	19%
T 1047	56f	7	pneum.	H	9	chorea	P	4.3[1.4]	5.2[1.9]	21%
T 940	36f	6	f.u.o.	H, per.	10	chorea	P	10.1[1.6]	12.6[2.4]	25%
T 874	65m	2	pneum.	H(1)	10	chorea	P	9.1[2.6]	12.0[2.4]	31%
T 952	29m	17	pneum.	H	7	chorea	P	7.1[4.8]	9.9[7.1]	39%
T 887	62f	18		H	12	chorea	P	6.2[2.0]	10.9[5.3]	43%
Mean ± s.e.	54 ± 4	9.3 ± 2						9.4 ± 1.6[2.8]	11.3 ± 1.7[3.3]	20.3% ± 4%
	64.2 ± 4	Mean ± s.d. of controls, Alzheimer's and Huntington's:								21.6% ± 3.7%
Schizophrenia										
CS 108	74f	28	cancer	Tr(18)	22	voices	CN	11.2[5.3]	11.2[4.1]	0%
CS 109	44f	34	AHF	Fs (~5)	26		P	14.5[3.4]	14.5[2.9]	0%
CS 112	84f	37	CHF	th(9), c(4)	48	voices	P + CN	13.4[2.2]	13.4[1.3]	0%
CS 131	67m	30	CHF	H(4), th(1)	24	visions	CN	22.5[8.8]	22.3[7.8]	0%
CS 135	72m	15	MI	Fs(4)	39	voices	P + CN	20.5[7.4]	20.0[7.4]	0%
CS 147*	95f	46	AHF	th(10)	63	voices	P	30.5[6.1]	27.2[4.4]	0%
CS 159*	20m	37	suicide	F(1)	1	paranoid	P + CN	22.2[6.4]	22.1[5.2]	0%
T 957	52f	21	pneum.	H (~15)	34	TD	P	7.0[1.3]	6.7[1.2]	0%
CS 165	65f	<24	MI	Tr(12)	40	confused	P + CN	21.2[8.3]	21.3[6.0]	0.5%
T 877*	75f	12	cancer	N		jaundice	P	23.5[4.4]	23.7[4.6]	0.9%
T 808*	60m	13	CVA	doxepin		catatonia	P	14.2[1.2]	14.4[1.2]	1.4%
CS 104	56m	28		c(24)	26		P	32.8[9.6]	33.4[8.7]	1.8%
CS 107	35m	51	MI	c(>5)	15	voices	P + CN	22.6[5.6]	23.0[3.9]	1.8%
CS 127	81f	40		thiop.(y?)	51	hallucin.	CN	20.1[3.2]	20.5[2.1]	2.0%
CS 169*	68m	38	CHF	off > 4 y	>40	aggression	P + CN	7.9[3.0]	8.1[2.2]	2.5%
T 1081	49m	17	suicide	H (~1)			P	28.2[4.0]	29.0[4.4]	3.0%
CS 117	64f	32	pneum.	H(2), Fp(4)	34	voices	CN	13.5[3.1]	14.0[2.0]	3.7%
T 499*	63m	4	GI inf.	F (~3)	~20	delusions				
T 802*	81m	2.5	CHF	procainam.	51	catatonia				
V 375-83*	81f	~12	CHF	F	15	paranoid				
V 433-88*	77f	12	pulm.	Fp (~10?)	40	halucin.				
Mean ± s.e.	64.9 ± 4	25 ± 3						19.2 ± 1.8[4.9]	19.1 ± 1.8[4.1]	1% ± 0.3%

Abbreviations and symbols (items missing in this table were not in patient case records): *, tissue used for [³H]ketanserin or [³H]SCH 23390 binding; accid., accident; AHF, acute heart failure; B, receptor density; c, chlorpromazine; chloram, chlorambucil; CN, caudate nucleus; CHF, congestive heart failure; CS, tissue from Cambridge Brain Bank, University of Cambridge; CVA, cerebrovascular accident (stroke); dig., digoxin; dipyr. id., dipyr. id.; F, fluphenazine; Fp, flupenthixol; Fs, fluspirilene; f.u.o., fever of unknown origin; GI inf., gastrointestinal infarct; Gpp[NH]p, guanylimidodiphosphate present (200 µM final); H, haloperidol; [³H], tritium; hallucin., hallucinations; hyperten., hypertension; K, dissociation constant; L, loxapine; MI, myocardial infarction; N, neuroleptic of unknown type; P, putamen; per., perphenazine; pneum., pneumonia; PM, postmortem delay (death-to-brain-freezing interval); procainam., procainamide; pulm., pulmonary oedema; T, tissue from Canadian Brain Tissue Bank, Toronto; TD, tardive dyskinesia; th, thioridazine; thiop., thiopropazine; TP, thiethylperazine; Tr, trifluoperazine; V, tissue from Lainz Hospital, Vienna.

the insensitivity of [³H]raclopride binding to guanine nucleotide in the schizophrenia tissues could not readily be attributed to the premortem use of neuroleptics, because most of the Huntington and Alzheimer patients had also received neuroleptics. Although it was not possible to retrieve the life-long doses from the incomplete case records, it was possible to estimate that both the Huntington and the schizophrenia patients received neuroleptics for an average of about 8-9 years (see Table 1 for ranges of illness and drug years). The average neuroleptic dose for schizophrenia outpatients on a long-term basis is 410 mg chlorpromazine equivalents²⁷, which is the average dose for Huntington patients.

The addition of 400 to 800 nM exogenous dopamine to the incubation tubes containing the schizophrenia tissue depressed the apparent density of [³H]raclopride, as previously reported⁹. This effect, however, of exogenous dopamine could be reversed by 200 µM guanine nucleotide. This indicates that the guanine nucleotide sensitivity of dopamine D2 receptors in schizophrenia can be restored by high concentrations of exogenous dopamine. The endogenous dopamine in postmortem schizophrenia tissues is normal¹³. Thus, it seems that the G protein subunits are still operative in the postmortem tissues, but there may be an abnormal regulation or stoichiometry of these subunits. The influence of postmortem delay (Table 1) is less obvious. Our previous

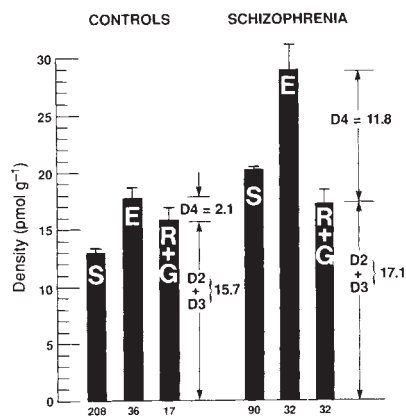


FIG. 2 Summary of present and previous^{12,18} data, indicating that dopamine D2 receptors in schizophrenia are insensitive to guanylimidodiphosphate, and that the density of dopamine D4 receptors is elevated sixfold in schizophrenia. The density of [³H]spiperone (S) sites¹², included for comparison, averaged 12.9 ± 0.2 pmol g⁻¹ in control tissues ($N=208$ brains; ages 20 to 88 yrs), and 20.2 ± 1.2 pmol g⁻¹ in schizophrenia striata ($N=90$). The density of [³H]emonapride (E) sites (which includes dopamine D2, D3 and D4 receptors) averaged 17.8 ± 1.0 pmol g⁻¹ in control brains ($N=36$) and 28.9 ± 2.2 pmol g⁻¹ ($N=32$) in schizophrenia (Table 2). In the presence of guanine nucleotide (G), the density of [³H]raclopride (R) sites (which only includes dopamine D2 and D3 receptors, because raclopride has a low affinity for D4) was 15.7 ± 1.2 pmol g⁻¹ ($N=17$; Tables 1 and 2) in control striata, and 17.1 ± 1.3 pmol g⁻¹ in schizophrenia striata ($N=32$; Tables 1 and 2), an increase of ~10%. The density of dopamine D4 receptors, however, measured as the difference between the [³H]emonapride and [³H]raclopride (with nucleotide) densities, increased by sixfold in schizophrenia.

work found that the densities of human¹² and rat (T. Lee and P.S., unpublished results) dopamine receptors were constant *in situ* for 48 hours postmortem at 4 °C. The schizophrenia tissues studied here averaged 25 h postmortem delay, compared to 9–13 h delay in the controls. Although it is possible that this difference in postmortem delay may account for the insensitivity of the schizophrenia tissues to guanylimidodiphosphate, the G protein subunits are still operative in these tissues, as noted above. Moreover, Huntington tissues with long delays of 17 or 18 h revealed a normal effect for guanine nucleotide (Table 1).

The results, together with the fact that raclopride has an extremely low affinity for dopamine D4 receptors¹⁶ (with a dissociation constant of $2,000 \pm 250$ nM; P.S. and H.H.M.V.T., unpublished results), permit an important analysis of other data^{12,18}, including the new data in Table 2, as summarized in Fig. 2. This indicates that the average density of [³H]spiperone sites is 12.9 pmol g⁻¹ in control human striata, and 20.2 pmol g⁻¹ in schizophrenia striata, an increase of 56%, as previously reported¹². It is not possible, however, to compare such [³H]spiperone data with [³H]raclopride data, because the cloned dopamine receptor binds roughly two [³H]raclopride molecules (or two [³H]emonapride molecules) for each [³H]spiperone molecule²⁸, presumably an indication of monomer-dimer formation of dopamine receptors. [³H]Emonapride labels dopamine D2, D3 and D4 receptors (with dissociation constants of 0.09 nM for D2, 0.3 nM for D3 (P. Sokoloff, personal communication) and 0.147 nM for D4 (P.S. and H.H.M.V.T., unpublished results), whereas [³H]raclopride only labels dopamine D2 and D3 receptors (with the same dissociation constant of 1.8 nM^{16,19}).

Hence, the difference between the [³H]emonapride and [³H]raclopride densities (in the presence of guanine nucleotide to remove the interfering effect of endogenous dopamine on the binding of [³H]raclopride) reveals the dopamine D4 density to

be 2.1 pmol g⁻¹ in control striata and 11.8 pmol g⁻¹ in schizophrenia, roughly a sixfold elevation (Fig. 2). Furthermore, if the densities of the dopamine D4 receptors from the control and Parkinson tissues are averaged (1.8 pmol g⁻¹; $n=22$ brains) and considered as a control value, the dopamine D4 receptor density in schizophrenia is elevated by 6.6-fold. The combined density of dopamine D2 and D3 receptors, however, as labelled by [³H]raclopride (in the presence of guanine nucleotide), only rises by about 10% (Fig. 2), in agreement with the *in vivo* data reported by Farde *et al.*¹⁵, using [¹¹C]raclopride. (One month treatment of rats with high doses of haloperidol elevated dopamine D4 receptors in rat striatum by a maximum of 1.9-fold, compared to 600% elevation found for dopamine D4 receptors in schizophrenia; P.S. and H.H.M.V.T., manuscript in preparation. Moreover, preliminary results for the dopamine D4 receptor densities in the brain striata from Huntington and Alzheimer

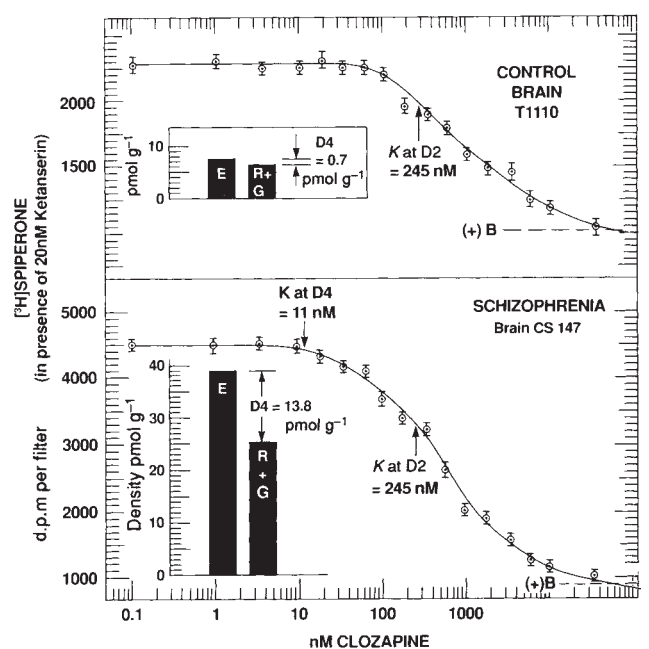


FIG. 3 Top, in control human brain striatum (T1110) clozapine recognized only one [³H]spiperone binding site, the dopamine D2 receptor site, because the dopamine D4 receptor density was very low, less than 10% of the D2 receptor density. Because the affinity of clozapine for the dopamine D4 receptor is enhanced in the absence of NaCl (ref. 31), the NaCl was omitted here. To preclude [³H]spiperone from binding to serotonin S2 receptors, 20 nM ketanserin was present. The dissociation constant for clozapine ($K=245$ nM) was derived from these data by means of the program LIGAND^{16,23,24,29}, where the dissociation constant of [³H]spiperone was 54 pM (in the absence of NaCl) and the final concentration of [³H]spiperone was 233 pM. Bottom, in schizophrenia brain striatum CS147, however, the dopamine D4 density was elevated to 13.8 pmol g⁻¹, and clozapine resolved the binding of [³H]spiperone to D2 and D4 receptor sites with dissociation constants of 245 nM (72% of total binding sites) and 11 nM (28% of total binding sites), respectively. Conditions were identical to those in the top panel, where NaCl was omitted and 20 nM ketanserin was present. The two dissociation constants for clozapine were derived by LIGAND, using dissociation constants of 54 pM and 81 pM for [³H]spiperone at the dopamine D2 and D4 receptors, respectively, in the absence of NaCl, and using a final [³H]spiperone concentration of 233 pM. In the absence of NaCl, it was necessary to use $1 \mu\text{M}$ (+)butaclamol [(+)B] to define nonspecific binding. These are representative data from three independent experiments. Vertical bars indicate s.d. for the replicate filters. The findings in the presence of NaCl were qualitatively similar, with the dissociation constants of clozapine being 300 nM and 33 nM at the dopamine D2 and D4 receptors, respectively (data not shown).

TABLE 2 Dopamine D4 receptor densities

	[*H]YM pmol g ⁻¹	[*H]Raclo + G pmol g ⁻¹	D4 pmol g ⁻¹	N?		[*H]YM pmol g ⁻¹	[*H]Raclo + G pmol g ⁻¹	D4 pmol g ⁻¹	N?
Controls					Schizophrenia				
T309	16.8	14.4	2.4	N-	CS104	44.8	33.6	11.2	N+
T360	21.7	21*	0.7	N-	CS107	28.1	21.2	6.9	N+
T362	20.0	16.1	3.9	N-	CS108	21.6	11.0	10.6	N+
T560	21.9	17.7	4.2	N-	CS109	29.9	14.5	15.4	N+
T1028	11.3	9.4	2.9	N-	CS112	21.5	12.6	8.9	N+
T1030	15.6	14.3	1.3	N-	CS117	16.3	15.0	1.3	N+
T1031	19.0	18.4	0.6	N-	CS127	24.8	20.4	4.4	N+
T1049	9.3	8.1	1.2	N-	CS131	32.2	22.3	9.9	N+
T1050	10.8	8.3	2.5	N-	CS135	21.7	17.8	3.9	N+
T1051	22.4	20.1	2.3	N-	CS147	39.2	27.2	12	N+
T1084	24.8	22.3	2.5	N-	CS165	24.5	20.1	4.4	N+
T1136	16.2	15.9	0.3	N-	CS169	10.5	8.0	2.5	N-
Mean ± s.e. (n = 12)	17.5 ± 1.4	15.5 ± 1.3	1.2 ± 0.3		L346	39.4	17.1	22.3	?
Parkinson's disease									
n = 6†	20.5	19.4	1.1		L477	16.2	6.3	9.9	N+
T709	15.1	13.6	1.5		L1651	9.8	5.8	4	N+
T817	20.0	17.0	3		T289	27.6	16.2	11.4	N+
T836	20.4	17.8	2.6		T321	16.5	5.6	10.9	?
T1076	13.6	12.0	1.6		T348	42.5	28.9	13.6	N+
Mean ± s.e. (n = 10)	19.2 ± 0.8	17.7 ± 0.8	1.5 ± 0.2		T499	20.1	14.3	5.8	N+
Average of controls and Parkinson's:			1.8 (n = 22)		T524	24.3	13.3	11	N-
Alzheimer's disease									
T491	9.2	7.4	1.8	N-	T751	21.0	13.0	8	N-
T528	17.0	14.4	2.6	N-	T802	28.0	7.4	20	N-
T538	16.6	10.3	6.3	N+	T808	20.0	14.4	5.6	N-
T547	12.8	11.5	1.3	N-	T894	43.5	22.2	21.3	N+
T548	21.8	15.8	6	N+	T1081	51.9	28.1	23.8	N+
T624	15.1	11.7	3.4	N+	T1149	35.7	25.3	10.4	N+
T634	18.0	15.5	2.5	N+	V185-80	24.3	12.8	11.5	N-
T646	19.6	16.9	2.7	N-	V75-82	23.4	17.9	5.5	N+
T652	19.2	14.7	4.5	N-	V374-83	59.5	22.7	36.8	N+
T669	37.0	36.0	1	N+	V375-83	55.2	20.4	35.2	N+
T680	9.4	8.5	0.9	N+	V570-84	19.6	13.5	6.1	N+
T695	26.3	24.3	2	N-	V433-88	37.5	22.8	14.7	N+
T697	9.4	7.3	2.1	N+	Mean ± s.e. (n = 32)	29.1 ± 2.2	17.3 ± 1.3	11.9 ± 1.5	
T713	20.0	17.0	3	N-					
T716	22.7	19.5	3.2	N+					
T717	17.2	14.8	2.4	N-					
T719	14.6	13.8	0.8	N+					
Mean ± s.e. (n = 17)	18.0 ± 1.6	15.3 ± 1.6	2.7 ± 0.4						

Dopamine D4 receptor densities. Abbreviations and symbols as in Table 1, with the following additions: N+, N- or ? indicate the long-term premortem use, non-use or unknown use of neuroleptics, respectively; † indicates data from ref. 18; * indicates that the density of [³H]raclopride binding sites in this tissue was 25% higher than that without guanine nucleotide; L, tissue from the National Neurological Research Bank, Wadsworth Medical Center, Los Angeles; n = number of different human brains (putamina) analysed; YM, emonapride; Raclo, raclopride.

patients treated for many years with neuroleptics also revealed a maximum of only 1.9-fold elevation.)

This analysis (Fig. 2) resolves the apparent discrepancy where the density of [¹¹C]methylspiperone-labelled sites¹⁴ (D2, D3 and D4), but not that for [¹¹C]raclopride-labelled sites¹⁵ (D2 and D3), was elevated in the schizophrenia striatum.

To obtain independent evidence that dopamine D4 receptors are increased in schizophrenia and that these sites have a D4 pharmacology (for example, more sensitive to clozapine), we competed clozapine versus [³H]spiperone in control brain striatum T1110 and in schizophrenia striatum CS147. The dopamine D4 receptor density in this control striatum was very low, namely 0.7 pmol g⁻¹ or less than 10% of the D2 density (Fig. 3), such that clozapine did not detect this site but only recognized the D2 sites (Fig. 3, top). In the case of the schizophrenia striatum, however, the D4 density was 13.8 pmol g⁻¹, sufficiently high for clozapine to resolve the binding of [³H]spiperone to dopamine D2 and D4 sites (Fig. 3, bottom), with clozapine dissociation constants of 245 nM and 11 nM, respectively, at these two sites. (Although we also tried to measure the D4 pharmacology by using [³H]emonapride in the presence of 200 nM raclopride, this

method markedly inhibited the binding of [³H]emonapride to the control tissue, making comparisons with the schizophrenia tissues difficult.) We are currently developing a [³H]ligand selective for dopamine D4 receptors such as to measure directly any elevation in these receptors.

The elevation of the binding of [³H]raclopride by guanine nucleotide in control tissues has been previously reported^{9,20,22,23,32}. The present findings of 21% elevation (Table 1 and Fig. 1) almost quantitatively agree with ref. 32 where they reported 29% to 38% elevation in the binding of [³H]raclopride to control human striata. Guanine nucleotide normally increases the binding of [³H]raclopride, as well as that of [³H]SCH 23390 binding, by converting the receptors to their agonist low-affinity state for dopamine^{29,30}, permitting [³H]raclopride or [³H]SCH 23390 to bind⁹. The insensitivity of [³H]raclopride binding in the schizophrenia tissues to guanine nucleotide (as well as the lowering of [³H]SCH 23390 binding by guanine nucleotide) indicates possible abnormalities at some point or pathway between the dopamine receptors and the G protein subunits⁹. At present, however, there is no particular reason for considering this insensitivity to guanine nucleotide, as well as the dopamine D4 recep-

tor elevation, as being specific to schizophrenia, because Parkinson-diseased tissue, which has no endogenous dopamine, is also insensitive to guanine nucleotide. If the nucleotide insensitivity is related to the psychotic process, then it may be expected that other diseases associated with psychosis may reveal insensitivity to guanine nucleotide and may also reveal elevated dopamine D4 receptors. □

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Ether-à-go-go encodes a voltage-gated channel permeable to K^+ and Ca^{2+} and modulated by cAMP

Andrea Brüggemann^{*†}, Luis A. Pardo^{*‡},
Walter Stühmer^{*§} & Olaf Pongs[†]

^{*} Max-Planck-Institut für experimentelle Medizin, Hermann-Rein-Strasse 3, D-37075 Göttingen, Germany
[†] ZMNH, Institut für Neuronale Signalverarbeitung, Martinistrasse 52, D-20246 Hamburg, Germany
[‡] Departamento de Biología Funcional, Universidad de Oviedo, E-33006 Oviedo, Spain

THE *Drosophila ether-à-go-go (eag)* mutant is responsible for altered potassium currents in excitable tissue¹. These mutants exhibit spontaneous, repetitive firing of action potentials in the motor axons of larval neuromuscular junctions². The *eag* gene encodes a polypeptide³ that shares sequence similarities with several different ionic channel proteins, including voltage-gated potassium channels³, an inward rectifier^{4,5} as well as cyclic-nucleotide-gated channels⁶. These formal similarities in the derived primary sequences indicate that *eag* polypeptides might express a new type of ion channel. Here we report the expression by *eag* RNA in *Xenopus* oocytes of such a channel which incorporates properties of both voltage- and ligand-gated channels. The permeability of these *eag* channels to potassium and calcium is dependent on voltage and cyclic AMP. The ability to mediate potassium-outward and calcium-inward currents endows this channel with properties likely to be important in the modulation of synaptic efficiency in both central and peripheral nervous systems.

We used available *eag* complementary DNA sequence information to design *eag*-specific primers for transcription of first-strand cDNA from poly(A)⁺ RNA isolated from adult *Drosophila melanogaster* heads. The cDNA served as a template in the polymerase chain reaction (PCR) for *eag* cDNA amplification and cloning (see legend to Fig. 1). When injected into *Xenopus*

oocytes, *eag* messenger RNA induced the expression of non-inactivating voltage-dependent outward currents. Macroscopic currents recorded with a conventional two-microelectrode voltage clamp had a threshold for activation between −40 and −30 mV (Fig. 1a, b). The currents were biphasic, having an initial fast-rising phase followed by a slower second component ($N > 100$). *I-V* relationships measured in inside-out patches are virtually identical (Fig. 1d). However, the biphasic rising phase was absent, and a partly inactivating current was observed ($N > 100$; Fig. 1c). This inactivation has a fast and a slow component, with time constants of 15.2 ms and 470 ms at a test potential of +40 mV. The fast component represents about 20% of the total current amplitude. Inactivation was never complete under the experimental conditions. A single-channel conductance of 4.9 pS was determined by non-stationary noise analysis⁷ using over 500 test pulses to each test potential of 0, +30 and +60 mV. Electrophysiological and pharmacological features of *eag* channels are shown in Table 1. Unlike most known K^+ -channels⁸, *eag* channels are resistant to high concentrations of 4-aminopyridine (4-AP).

The slope of the plot of the reversal potential against external K^+ concentration was 55 mV, as expected for a highly selective K^+ channel. The amplitude of the outward current increased when the extracellular K^+ concentration was raised, like the effects of external K^+ that have been described for wild-type Kv1.4 (ref. 9) and certain mutant *Shaker* channels¹⁰. The relative permeabilities to different ions were determined by measuring the reversal potentials in bi-ionic conditions (115 mM of the chloride salt of the test ion and 1.8 mM EGTA outside, versus 100 mM KCl and 10 mM EGTA inside) in outside-out patches. The results (Table 1) indicate that *eag* channels are not very permeable to Na^+ or Li^+ , but are unexpectedly permeable to NH_4^+ and Cs^+ . The sequence of permeabilities is $K^+ > Rb^+ > NH_4^+ > Cs^+ \gg Na^+ > Li^+$ (Table 1), which corresponds to Eisenman's series IV (ref. 11).

The slow-rising phase of *eag* currents seen only under two-electrode voltage-clamp (Fig. 1a) was reduced in the presence of the chloride-channel blockers niflumic acid¹² ($N > 30$; Fig. 2a) or mefenamic acid (legend to Fig. 2a) in Ca^{2+} -free bathing solutions (not shown), or on chelation of intracellular Ca^{2+} with BAPTA ($N = 6$; Fig. 2b). It reappeared under conditions that allowed Ca^{2+} -dependent chloride currents, such as washing out niflumic acid or adding Ca^{2+} to the bath. These results suggested

§ To whom correspondence should be addressed.

TABLE 1 Electrophysiological and pharmacological properties of *eag* channels

Permeability Test ion	K ⁺	Rb ⁺	Cs ⁺	NH ₄ ⁺	Na ⁺	Li ⁺
Relative permeability	1	0.75 ± 0.11	0.42 ± 0.14	0.25 ± 0.08	0.11 ± 0.06	0.08 ± 0.06
Kinetic parameters	Activation (+40 mV) τ 1.71 ± 0.29 ms	Deactivation (-100 mV) τ_1 73 ms τ_2 3 ms	$V_{1/2}$ -27.9 ± 10.29 mV	Inactivation (+40 mV) τ_1 15.2 ms τ_2 470 ms		Recovery (-100 mV) 270 ms
Pharmacology IC ₅₀	TEA 33 ± 11 mM	4-AP >100 mM	Quinine 0.7 ± 0.3 mM	Quinidine 0.4 ± 0.15 mM		

Permeability ratios were determined from the reversal potential obtained in outside-out patches exposed to 100 mM KCl, 10 mM EGTA, 10 mM HEPES, pH 7.2, as internal solution, and 115 mM of the test cation (chloride), 1.8 mM EGTA, 10 mM HEPES, pH 7.2, on the inside. All the kinetic parameters were measured in inside-out patches, with 100 mM KCl, 10 mM EGTA, 10 mM HEPES, pH 7.2, as internal solution and 115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, 10 mM HEPES, pH 7.2, as external solution. $V_{1/2}$ refers to the potential at which the half-maximal conductance was obtained, and was determined by fitting a Goldman-Hodgkin-Katz function to the I - V relationships. The pharmacology of the channel was determined using two-electrode voltage clamp.

that the slow-rising phase of outward currents was due to a calcium influx in *eag*-expressing *Xenopus* oocytes causing activation of a Ca²⁺-dependent chloride conductance. A rough estimate of the permeability ratio (Ca²⁺ to K⁺) of less than 1/10 can be obtained from reversal potential shifts induced by Ca²⁺ under niflumic acid.

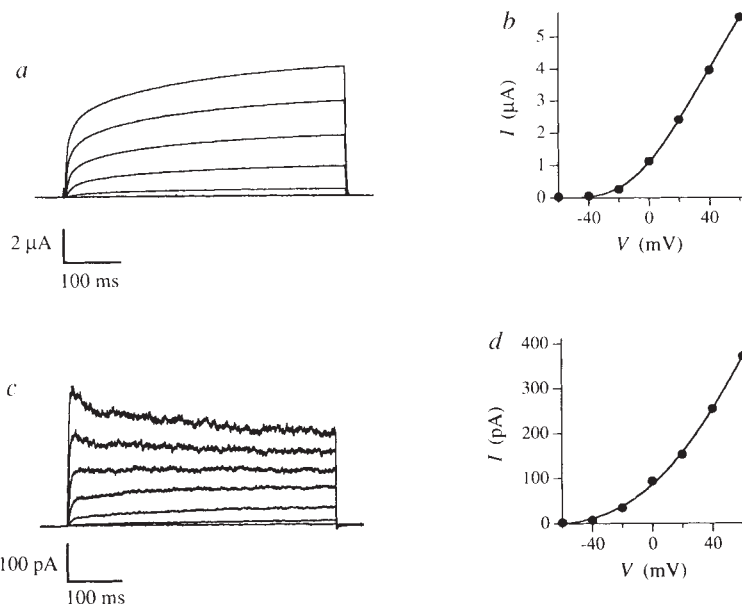
Cadmium ion at 1 mM, which blocks endogenous voltage-gated *Xenopus* Ca²⁺-channels¹³, did not affect the amplitude of *eag*-mediated outward currents ($N=10$; Fig. 2c). Only simultaneous or subsequent addition of niflumic acid blocked the slow-rising phase of *eag* outward current. Using a tail-current protocol, we investigated the calcium influx in more detail (Fig. 2c). The Ca²⁺ inward currents were blocked to the same extent by 30 mM tetraethylammonium (TEA), as were the *eag*-mediated K⁺ outward currents. The amplitudes of the tail currents (normalized to the outward current) were larger in 115 mM Ca²⁺ than in 115 mM Na⁺ outside. Direct evidence for Ca²⁺ influx through *eag* channels using the Ca²⁺-sensitive fluorescent dye Fluo-3 is shown in Fig. 3. These results indicate that the expres-

sion of *eag* RNA in *Xenopus* oocytes leads to the formation of a new kind of voltage-activated ion channel which is able to evoke a potassium efflux as well as a simultaneous calcium influx.

The *eag* and cyclic-nucleotide-gated channel polypeptides share, over a stretch of ~500 amino acids, 24% identical residues and 20% conservative substitutions, including a consensus cyclic-nucleotide-binding site⁶. Its possible function in *eag* channel activity was tested. The addition of either 8-Br-cAMP or 8-Br-cGMP to the bath solution increased the amplitude of *eag* outward currents. The threshold of their activation was shifted to more negative potentials ($N>30$; Fig. 4a). The effect of 8-Br-cAMP, but not that of 8-Br-cGMP, persisted in the presence of the nonspecific protein-kinase inhibitor H-7 (1-(5-isoquinolylsulphonyl)-2-methylpiperazine; $N=3$; data not shown). Accordingly, rapid application of 2 mM cAMP (but not of cGMP or ATP) to inside-out patches of *eag*-expressing *Xenopus* oocytes produced a significant increase in outward-current amplitude ($N=10$; Fig. 4b). This increase was rapidly reversible on perfusion with normal (intracellular) bathing solution lacking

FIG. 1. Currents elicited by depolarizing steps in *Xenopus* oocytes expressing *eag* mRNA. The depolarization ranged from -60 mV to +60 mV from a holding potential of -80 mV. a, Currents measured using a two-electrode voltage clamp and, c, in an inside-out patch. The extracellular solution was always normal frog's Ringer. Current-voltage relationships of the outward-currents recorded in a and c are plotted in b and d, respectively.

METHODS. Poly(A)⁺ RNA was prepared from adult *Drosophila melanogaster* heads as described¹⁸. First-strand cDNA synthesis¹⁹ was primed with ATTATTGCTCCTGGCTGGAG as primer, which is complementary to the 3' untranslated sequence of the published *eag* cDNA sequence³; *eag* cDNA was amplified by PCR with the following primers spaced along the sequence. The forward primers were: AAGCCACGCAAGATCGATTC (98-117); ATATGATGTCCTTAAGCGCG (1,077-1,096); GCACCTTCATGATGTCGCACTCGG (2,219-2,241). The reverse primers were: TCCTGTCGGTACTGTGGCAT (1,122-1,141); GTGACGATGAAGCATAGGCT (2,302-2,321); and TTCGCTCCTCCCGATCGTCGTG (4,014-4,036). Amplified fragments were subcloned into Bluescript KS⁺ for sequencing both strands²⁰. In comparison to the previously published *eag* cDNA sequence³, the open reading frame derived from our cDNA was two amino acids (Y 68 and V 69) shorter because nucleotides 665 to 670 were missing. In our *eag* cDNA sequence, we detected a different nucleotide at four positions (2,174 A→G; 2,768 C→T; 3,263 A→G; 3,608 G→A), which altered the *eag* protein sequence at amino-acid residues T at 571 to A, L at 769 to F, S at 967 to A, and E at 1,049 to K, respectively. We found a different nucleotide at two additional positions (C at 1,411 to T, and T at 1,980 to C) that did not alter the protein sequence.



We detected the differences in several independent PCR-amplified *eag* cDNA fragments. We concluded that the sequence differences were due to fly-strain polymorphism (CantonS versus OregonR) rather than to transcriptional errors of the polymerase during PCR. *In vitro* transcription was performed using the expression vector pAKS2 as described²¹.

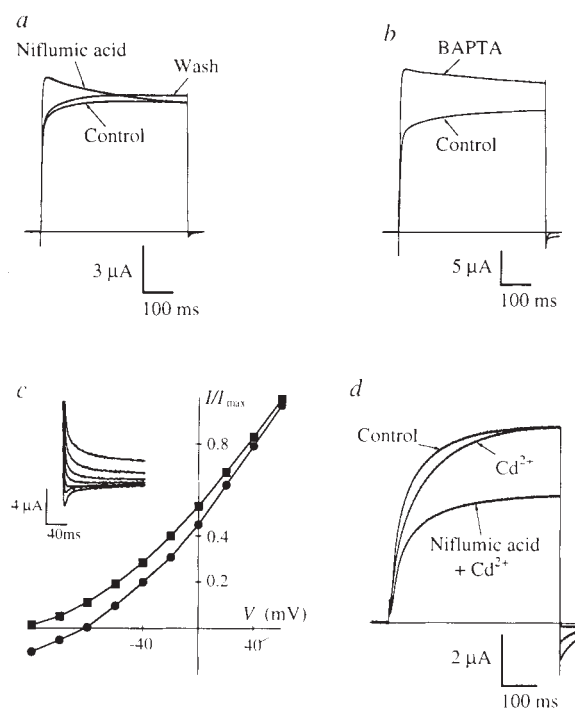


FIG. 2 The *eag* channels are permeable to calcium. *a*, The biphasic kinetics of the current traces in two-electrode voltage clamp ('control') disappeared in the presence of 500 μ M niflumic acid and an inactivation component became apparent ('niflumic acid'). After washing, control kinetics reappeared ('wash'). Using saturating concentrations of mefenamic acid (500 μ M), which also blocks Cl⁻ currents, the slow-

rising phase is abolished but not reversed (data not shown), indicating that saturating concentrations of mefenamic acid are not as efficient as niflumic acid in blocking Cl⁻ currents. But, in contrast to niflumic acid, mefenamic acid does not lead to an increase in currents. This might be because niflumic acid increases P_o in some K⁺ channels, an effect that is absent when mefenamic acid is used²². *b*, Chelation of intracellular Ca²⁺ by injection of BAPTA (1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; 50 nl at 100 mM, final concentration ~1 mM) also removes the biphasic activation kinetics and makes an inactivating component apparent. The increase in amplitude seems to be due to an increase of 'leak' current, which was also seen in control uninjected oocytes after injection of BAPTA. *c*, Ionic selectivity of *eag* channels measured by instantaneous *I/V* relationships in whole oocytes. Normalized current amplitude as a function of potential is shown for 115 mM Na⁺ plus 1.8 mM Ca²⁺ (■) or 115 mM Ca²⁺ (●); in both cases, Cl⁻ currents were blocked by 500 μ M niflumic acid. Tail currents ranging from -120 to 0 mV are shown in the inset for 115 mM Ca²⁺. The reversal potentials obtained were -130 mV for Na⁺ and -80 mV for Ca²⁺. *d*, 1 mM Cd²⁺, an effective blocker of endogenous oocyte Ca²⁺ channels¹², does not block *eag*-mediated currents. Notice the slow activation kinetics in the presence of Cd²⁺, which is a specific and direct effect of certain divalent cations on *eag* channels; *eag*-mediated currents are reduced only when 500 μ M niflumic acid is added with Cd²⁺.

METHODS. All two-electrode recordings were made using standard techniques²³. The external solution contained (in mM) 115 NaCl, 1.8 CaCl₂, 2.5 KCl, 10 HEPES, pH 7.2 (*a* and *b*), or 155 CaCl₂, 10 HEPES, pH 7.2 (*d*). These measurements were done by stepping from a holding potential of -80 mV to a depolarizing potential of +60 mV. Tail-current measurements were recorded (*c*) in the following external solutions: 115 mM test cation (chloride), 1.8 mM CaCl₂ (except for Ca²⁺ tails), 10 mM HEPES, pH 7.2, 500 μ M niflumic acid. Instantaneous *I/V* curves were determined using activation pulses to +80 mV for 30 ms, followed by test pulses ranging from -120 to +80 mV in 20-mV steps lasting 200 ms. Double exponential functions were fitted to the tail currents, and the extrapolated instantaneous current was normalized to the peak amplitude during the prepulse.

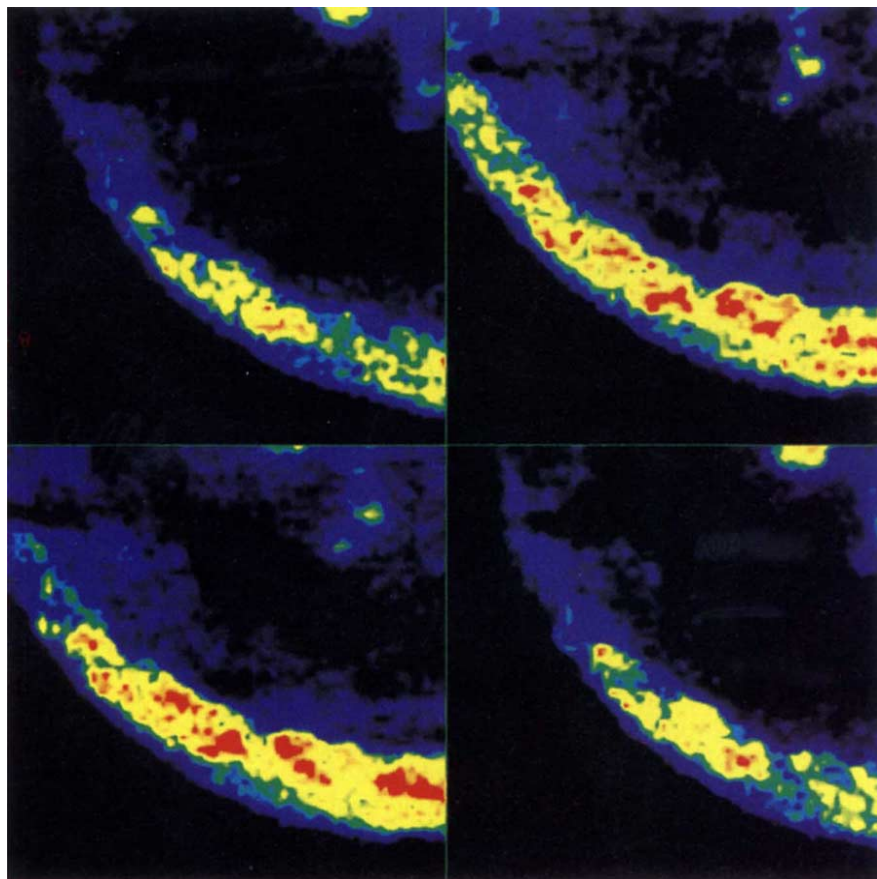
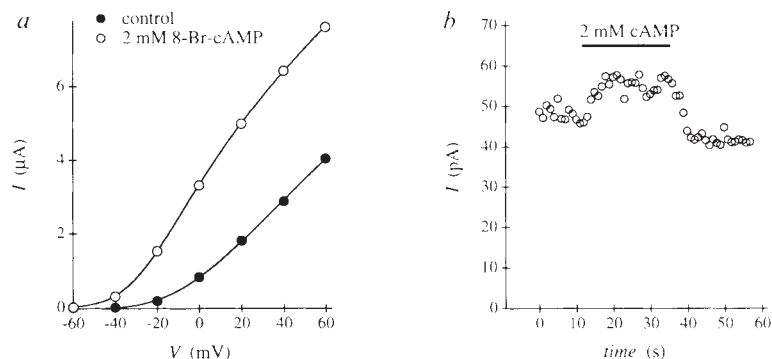


FIG. 3 Increase in fluorescence of the Ca²⁺-sensitive dye Fluo-3 following activation of *eag* channels. Electrical stimulation of the oocyte induces a reversible increase in fluorescent signal (top right) as compared to basal levels (top left). Addition of 1 mM CdCl₂ does not block the increase (bottom left), whereas 100 μ M quinine virtually abolishes the changes in fluorescence (bottom right).

METHODS. Oocytes expressing *eag* channels were injected with 50 nl 2 mM Fluo-3 (pentaammonium salt) in 150 mM KCl. The final concentration of the dye was ~20 μ M. Two to three hours after injection, oocytes were voltage-clamped, and Fluo-3 emission recorded using a Bio-Rad confocal microscope with a Krypton-Argon laser as excitation source (488 nm). The signal (>515 nm) was digitized and analysed using Bio-Rad interface and software. The recording chamber contained 75 mM CaCl₂, 2.5 mM KCl, 1 μ M BaCl₂ and 10 mM HEPES, pH 7.2. Addition of low concentrations of Ba²⁺ slows down the deactivation of *eag* channels (unpublished observations). The holding potential was set to -80 mV. The pulse protocol consisted of a depolarizing pulse to +60 mV lasting 200 ms, followed by a hyperpolarization to -120 mV during 500 ms. No increase in fluorescence was observed after 100 pulses applied to 15 uninjected oocytes under the same conditions.

FIG. 4 cAMP modulation of *eag* current. **a**, Addition of 8-Br-cAMP (○) to the bathing solution increased current amplitude with respect to control (●). Current amplitudes are shown as a function of test potential. Note that 8-Br-cAMP shifted the voltage-dependence of activation in the hyperpolarizing direction. Similar effects were observed using 2 mM 8-Br-cGMP. **b**, Fast application of 2 mM cAMP to the cytoplasmic side of inside-out patches containing *eag* channels increases the amplitude of the current. Outward currents were recorded after stepping from -80 mV holding to $+40$ mV test potential. Test-pulse duration was 50 ms and applied every second. After the twelfth test pulse, the patch was exposed for 25 s to a 2 mM cAMP containing standard intracellular solution (bar).



cAMP. These results may indicate a direct modulation of *eag* channels by cAMP.

Previously, a voltage-clamp analysis of *Drosophila* larval muscle showed that the amplitudes of several distinct outward currents (I_K , I_A , I_C) were reduced to various degrees in *eag* mutants^{1,14}. On the basis of these multiple effects, it was suggested that *eag* protein might coassemble with other K^+ -channel subunits into heteromultimeric A-type, delayed-rectifier and Ca^{2+} -activated K^+ -channels¹. Although our results cannot exclude this possibility, they offer a simple alternative explanation for the multiple electrophysiological alterations in *eag* mutants. Cloned *Shaker* channels have qualitatively similar electrophysiological properties in the *Xenopus* oocyte expression system and *in vivo*, although their pharmacological profiles are different¹⁵. Therefore *eag* channels probably also conduct outward potassium and inward calcium currents *in vivo*. Consequently, loss of these functional channels in *eag* mutants would be responsible for the *eag* phenotype, which has an apparent reduction in A-type, delayed-rectifier and Ca^{2+} -activated K^+ channel activities.

The existence of a link between voltage- and cyclic-nucleotide-gated ion channels has been proposed^{16,17}, and *eag* channels may represent such a link as they share properties with both families. The dual action of *eag* channels, which are activated by membrane depolarization and modulated by second messenger cAMP, could be involved in regulating membrane excitability in response to a change in cAMP concentration. Modulation of cyclic-nucleotide-gated channels is important for adaptive behavioural responses to light illumination or odour, and the modulation of *eag*-type channels may have similar implications for neural signal transduction in the central and peripheral nervous system. □

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Generating loss-of-function phenotypes of the *fushi tarazu* gene with a targeted ribozyme in *Drosophila*

Jack Jiagang Zhao* & Leslie Pick

Brookdale Center for Molecular Biology,
Mount Sinai School of Medicine, Box 1126,
One Gustave L. Levy Place, New York, New York 10029, USA

THE ability to isolate gene sequences and analyse their expression patterns has generated demand for mutations created to assess their biological functions. In *Drosophila melanogaster* this can be achieved by traditional mutagenesis, but this is time-consuming, labour-intensive and not always successful. Moreover, the functions of genes that are expressed several times during development are often obscured in the later stages because of disruptions caused by the absence of early gene function. Here we propose a new strategy to create conditional knock-out mutations using a targeted heat-inducible ribozyme. Ribozymes are catalytic RNA molecules that specifically cleave RNAs^{1–11} and are potentially useful for studying gene function during animal development because the expression of critical regulatory genes is usually low and their function is often dosage-dependent. The ribozyme can be delivered to a specific region or at a particular developmental stage using a region-specific or inducible promoter. The *Drosophila fushi tarazu* (*ftz*) gene is a good candidate for testing this approach^{12–17}. We generated transgenic flies carrying a ribozyme against the *ftz* gene. The two developmental phases of *ftz* function can be distinguished by timed induction of the ribozyme. Activation of the ribozyme in the blastoderm disrupts the *ftz* seven-stripe pattern and produces *ftz*-like pair-rule defects in larvae. The involvement of *ftz* in neurogenesis was verified by activation of the ribozyme during the early phase of formation of the central nervous system.

* Present address: Department of Neurobiology, The Scripps Research Institute, 10666 N. Torrey Pines Road, La Jolla, California 92037, USA.

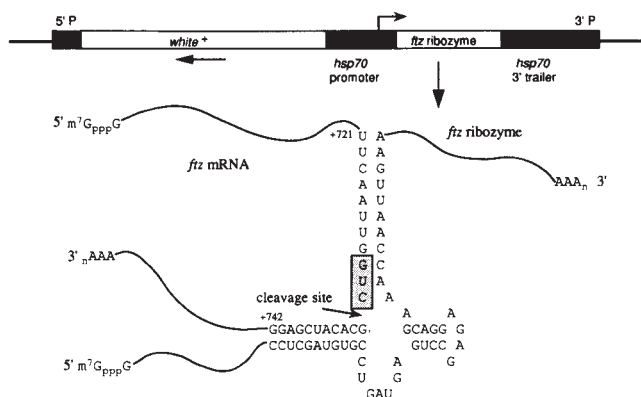


FIG. 1 The *ftz* ribozyme transgene. The *ftz* 'hammerhead' ribozyme, based upon plant viral RNA ribozymes², is under the control of a *Drosophila* heat-shock promoter. The transcripts consist of the *hsp70* 5' leader (~207 bases) and 3' trailer (~525 bases) with a poly(A) tail (not drawn to scale) to protect the ribozyme from degradation by intracellular RNases. This ribozyme is targeted to cleave *ftz* RNA between nucleotides +731 and +732, upstream of the homeobox¹², as shown in the lower part of the figure. Hammerhead ribozymes consist of a 24-nucleotide catalytic domain and a recognition motif complementary to a target RNA^{3,4}. As the only sequence requirement for cleavage of a target is that it should contain the trinucleotide sequence NUX (where N is G, A, U or C, and X is C, A or U)⁵, almost any RNA molecule can be targeted by appropriate ribozymes.

METHODS. A synthetic oligonucleotide containing the *ftz* ribozyme sequence 5'-CCTCGATGTGCCTGATGAGTCCGAGAGGACGAAACCAATTG-AA-3' with its complementary oligonucleotide was cloned into the *Sall* and *XbaI* sites of a pGEM7Zf⁻-derived vector which contains a fragment from HZ50PL (ref. 21), including 65 nucleotides from the 3' end of the *Escherichia coli lacZ* gene. The *ftz* ribozyme DNA, along with the *lacZ* sequence, was subcloned into the *EcoRI* and *XbaI* sites of a *Drosophila* P-element vector, CaSpeR-hs (ref. 22), and microinjected into *Df(1)w* eggs^{23,24}. Four independent germ-line transformants were obtained. They are viable under normal growth conditions. The transgenic flies carrying the *ftz* antisense fragment 5'-CCTCGATGTGCGACCAATTGAA-3' lacking the ribozyme catalytic domain, and the mutant *ftz* ribozyme 5'-CCTCGATGTGCCTAATGGGTCGAGAGGACGATACCAATTGAA-3', which has been mutated at three positions of the catalytic domain⁵ (underlined nucleotides) by base substitutes of G, A and A, respectively, were constructed in the same way.

We have constructed a *ftz* ribozyme that consists of a hammerhead catalytic centre flanked by sequences complementary to *ftz* messenger RNA between positions +721 and +742, near the splice site upstream of the *ftz* homeobox, and including a GUC cleavage site¹², embedded between the *hsp70* promoter and 3' untranslated sequences (Fig. 1). To determine whether the *ftz* ribozyme could efficiently block *ftz* protein synthesis in embryos, expression of the *ftz* ribozyme was induced between 2 and 2.5 h after egg laying (AEL). In the wild-type blastoderm, *ftz* protein is expressed in seven transverse stripes (Fig. 2a). After induction of the *ftz* ribozyme, *ftz* protein was dramatically decreased in embryos. This reduction was not uniform within a given batch of embryos (Fig. 2b, c); the stripes that appeared to be the most resistant to the ribozyme are those that emerge earliest (stripes 1, 2 and then 5; ref. 18, and Y. Yu and L.P., manuscript submitted). It is likely that small differences in the exact ages of embryos within the population account for the variable sensitivities observed.

Embryos homozygous for known *ftz* mutations die before hatching and lack alternate segments, as evidenced by loss of the odd-numbered abdominal denticle belts in the larval cuticle¹⁴. Expression of the *ftz* ribozyme at the blastoderm stage caused *ftz*-like pair-rule morphological defects in the resulting first instar larval cuticles (Fig. 3). The second thoracic segment (T2) and odd-numbered abdominal segments (A1, A3, A5 and A7 segments) were completely deleted in the most severely affected

cuticles (Fig. 3d). We also observed cuticles with less severe defects in the same preparations (Fig. 3b, c). Generally, A5 is the most sensitive to the *ftz* ribozyme, followed sequentially by A3, A7, A1 and T2. This range of phenotypes was also seen with a *ftz* temperature-sensitive mutant¹⁴. Furthermore, just as the A5 denticle was the most sensitive to loss of *ftz* activity due to the ribozyme, it was also the most difficult to rescue with altered *ftz* proteins¹⁹. Thus, the different sensitivities to the *ftz* ribozyme along the anterior-posterior axis are probably due to differential requirements for active *ftz* protein in different stripes.

Previous studies showed that antisense hybridization of ribozymes contributes in part to phenotypes in cultured cells^{9,11}. Two modified *ftz* ribozymes were made to assess the contribution of antisense effects to the observed *ftz* phenocopies. First, three point mutations were introduced into the catalytic domain of the ribozyme⁵ at positions known to be essential for ribozyme function ('mutant ribozyme'); second, the antisense portion of the ribozyme, lacking any catalytic domain, was synthesized ('antisense control'). Three different heat-shock conditions were tested in parallel for induction of the *ftz* ribozyme and each of the control constructs (see legend to Fig. 3). The effects of induction of the two control constructs were virtually identical ($\pm 1\%$).

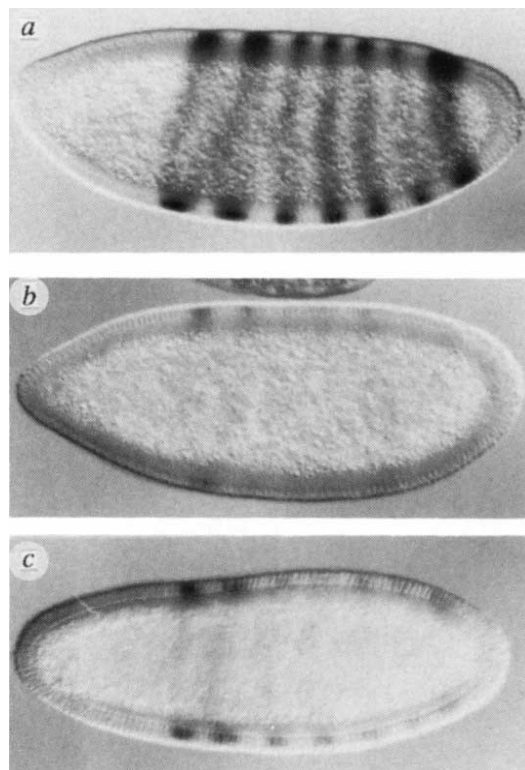
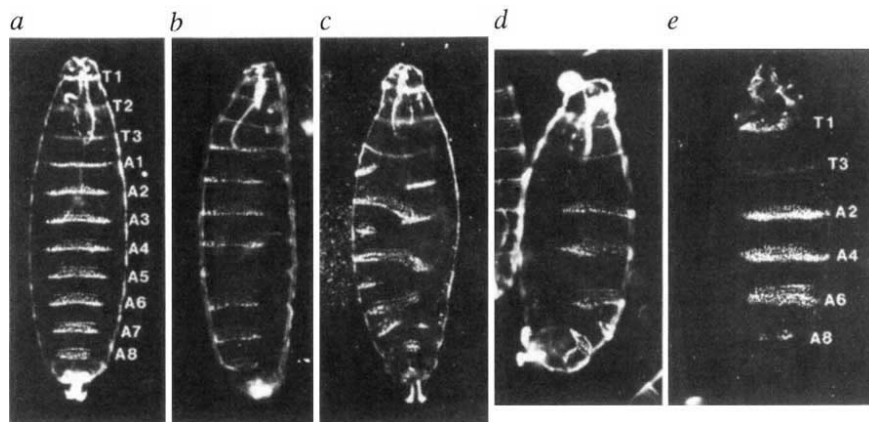


FIG. 2 Inhibition of *ftz* protein synthesis after induction of the *ftz* ribozyme in transgenic embryos. Embryos were given two short heat pulses at 2.0 to 2.5 hours AEL. Lateral views of whole-mount embryos are shown; anterior is left; dorsal is up. a, A wild-type embryo after heat shock; the *ftz* protein expression pattern is indistinguishable from a non-heat-shocked embryo. b and c, Embryos carrying the *ftz* ribozyme displayed various degrees of reduction of *ftz* protein: in b, most transverse stripes were missing; in c, *ftz* protein expression was much less than the wild type (compared with a); a few stripes, especially the first and second, were still detectable.

METHODS. Embryos were collected for 30 min and matured to 2.0–2.5 h at 25 °C, then treated with two heat pulses of 5 min in a 37 °C water bath, followed by 20 min recovery at 25 °C. The ages of embryos were verified after staining by the progression of nuclear elongation during blastoderm formation. Immunostaining was carried out by a modified standard protocol (T. Gutjahr and M. Noll, personal communication) using a monoclonal mouse anti-*ftz* antibody.

FIG. 3 Pair-rule phenotypes generated in *ftz* ribozyme transformants. *a*, Cuticular pattern of a wild-type first instar larva. *b*, The weakest pair-rule phenotype observed in ~11% of embryos after induction of the *ftz* ribozyme. Only the A5 denticle belt was missing. Other weak phenotypes, defined by partial gaps and/or fusions of more than one denticle belt, were observed in 8% of heat-shocked embryos. These weak phenotypes (total 19%) were also induced by antisense control constructs: about 16% of embryos carrying the mutated ribozyme, and ~15% carrying the antisense control. *c*, A moderate mutant phenotype having partial loss of A5, A3 and A1 denticle belts and fused A6 and A7 segments was observed in ~6% of embryos. These weak and moderate pair-rule phenotypes are similar to the abnormalities observed in *ftz* mutant embryos hemizygous for the *ftz*^{47ts} allele¹⁴ or *ftz*^{Rnl} allele¹⁵. Moderate phenotypes were only very rarely induced by antisense control constructs: 0.9% of embryos for the mutant ribozyme; 0.3% for the antisense control. *d*, A strong pair-rule phenotype was observed in ~5% of embryos after induction of the *ftz* ribozyme. A1, A3 and A5 segments were completely fused to T3, A2 and A4 respectively, whereas the A7 segment was partially deleted or fused. Strong phenotypes were never observed after induction of either the mutant ribozyme or the antisense control. *e*, A typical pair-rule



defect in the cuticle of a *ftz* mutant larva (homozygous for *ftz*⁹⁰⁹³ allele²⁵).

METHODS. In *a-d*, embryos were collected over one hour and allowed to develop at 25 °C. The 20-min heat shock was delivered to the embryos in a 37 °C water bath at 2.5–3.5 h AEL. In addition, two other conditions were tested: one 10-min heat shock delivered at 2.5–3.5 h AEL, and two 5-min heat shocks separated by a 20-min recovery at 25 °C. The cuticular preparation was based on ref. 26.

No strong *ftz*-like phenotypes (Fig. 3*d*) were obtained with either the mutant ribozyme or antisense control. Only ~10% of moderate phenotypes (Fig. 3*c*) could be attributed to antisense effects. Weaker phenotypes were induced frequently, however, by both the mutant ribozyme and antisense controls. Taking into account all three heat-shock conditions tested, the antisense contribution to the loss of two denticle belts was 51%; to the loss of A5 alone, 72%.

The *ftz* gene is also involved in nervous system development^{13,17}. To test the ability of the *ftz* ribozyme to distinguish the roles of *ftz* in segmentation and in neural development, we induced *ftz* ribozyme expression at 4.5–9 h AEL. The expres-

sion pattern of *eve* in the developing central nervous system was used as a marker to monitor neuronal abnormalities^{17,20}. In embryos expressing the *ftz* ribozyme, *eve* expression was greatly reduced in RP2 neurons (Fig. 4*c, d*), as was seen in *ftz* neurogenic mutants obtained with a *ftz* transgene expressed in stripes but not detectably in the nervous system¹⁷. Moreover, the *eve* pattern in the cluster of aCC, pCC and CQ neurons was also altered in some embryos (Fig. 4*c, d*). Thus, loss of *ftz* function seemed to affect the morphology and/or migration of these neurons. No segmentation defects were observed in cuticles of animals exposed to the ribozyme at this later stage of development.

These experiments demonstrate that induction of a *ftz*-specific ribozyme mimicked the effects of known *ftz* mutations. Moreover, the timed induction of the *ftz* ribozyme distinguished the functional properties of two separate phases of *ftz* expression: first, in segmentation and later, in neurogenesis. In addition, the range of phenotypes induced by the ribozyme allowed for an assessment of both 'hypomorphic' and 'amorphic' effects of a gene without the necessity for generating multiple mutations. To our knowledge, this is the first evidence that ribozymes can be used as tools to study animal development. Optimization of this

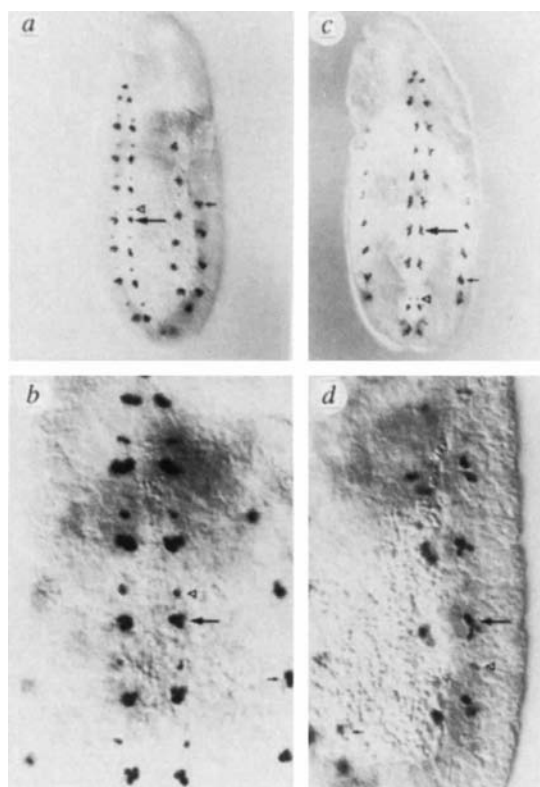


FIG. 4 Altered *eve* expression in the *Drosophila* CNS induced by the *ftz* ribozyme. The *eve* staining patterns are shown in wild-type embryos (*a, b*) and in the embryos expressing the *ftz* ribozyme (*c, d*) at age 8–9 h AEL with a whole-mount view (*a, c*) and a high-power view ($\times 76$; *b, d*). Embryos are viewed from the ventral side, with the anterior at the top. Triangles indicate RP2 neurons. The cluster composed of aCC, pCC, and CQ neurons is indicated by a long arrow; pericardial cells (non-neuron cells) are indicated by a small arrow. *a, b*, In a wild-type embryo, before finishing germ-band retraction, each hemisegment contains five neurons expressing equal amounts of *eve* protein²⁰. Among these, aCC, pCC and a pair of CQ neurons are clustered (indicated by a long arrow), whereas the RP2 neuron is located separately from the cluster (indicated by a triangle). In *c, d*, *eve* expression was disrupted in RP2 neurons after induction of the *ftz* ribozyme during neurogenesis. In most segments, no *eve* protein is detectable in RP2 neurons. In some segments, small amounts are still detectable (indicated by a triangle). Similar effects on *eve* expression were found in a *ftz* neurogenic mutant¹⁷. In the clusters, the level of *eve* expression was relatively normal but the pattern of the cluster was altered (compare *d* and *b*).

METHODS. Embryos aged to 4.5–5.5 h AEL were heat-shocked three times for 20 min at 37 °C with a 40-min interval at 25 °C for recovery between heat shocks. Treated embryos were fixed one hour after the final heat pulse and immunostained with anti-*eve* antibody.

technique should allow for improved efficiency in generating phenotypes for genes with unknown functions.

Note added in proof: We have recently demonstrated that the *ftz* ribozyme, but not the mutant ribozyme, can cleave *ftz* RNA in an *in vitro* reaction (A. S. Menzies and L.P., unpublished observation). □

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Convergent combination therapy can select viable multidrug-resistant HIV-1 *in vitro*

Brendan A. Larder, Paul Kellam & Sharon D. Kemp

Antiviral Therapeutic Research Unit, Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, UK

THE reverse transcriptase enzyme of human immunodeficiency virus type 1 (HIV-1) is the target for many inhibitors¹. Amino-acid substitutions in functional regions of the enzyme that abolish reverse transcriptase activity also prevent HIV-1 replication^{2,3}. But selection pressure by drugs such as AZT (3'-azido-3'-deoxythymidine, zidovudine)^{4–6}, ddI (2',3'-dideoxyinosine)^{7,8} and non-nucleoside reverse transcriptase inhibitors (NNRTIs)^{9–14} causes outgrowth of resistant variants due to non-lethal mutations in the enzyme^{7,9–16}. Reports of synergy^{17–19} and lack of cross-resistance between reverse transcriptase inhibitors (refs 7, 9, 10, 12–14, 17, 18, 20, 21), plus the reversal of AZT resistance by mutations induced by ddI⁷ and NNRTIs¹⁴, have indicated that specific drug combinations directed at reverse transcriptase might curtail resistance. Chow et al.²² extended this concept in a report that specific multiple combinations of resistance mutations in the reverse transcriptase can significantly impair HIV-1 replication. They concluded that evolutionary limitations may exist to prevent the emergence of multidrug resistance to inhibitors of reverse transcriptase²². We report here that HIV-1 co-resistant to AZT, ddI and the NNRTI nevirapine²³ can be readily selected in cell culture starting with dual AZT- and ddI-resistant virus. We found no evidence for 'replication incompatible' combinations of resistance mutations, although a mutation (M184→V) conferring oxathiolane-cytosine nucleoside resistance in reverse transcriptase^{24,25} completely suppressed AZT resistance in a triple-resistant background. These *in vitro* observations suggest that triple drug combination therapy might ultimately result in co-resistant HIV-1, although they do not preclude assessment of such combinations for treatment of HIV-1 disease.

Previously we found that resistance of HIV-1 to two reverse transcriptase inhibitors did not arise owing to a suppressed resistance to AZT^{7,14}. To explore the possibility of co-resistance, we determined the drug sensitivity of HIV-1 variants containing combinations of mutations in the commonly observed AZT-resistant genetic background of 41L/215Y¹⁶. When the ddI resistance mutation, 74V, was introduced into this virus by site-specific mutagenesis, the resulting strain (HIVRTMZ) was cross-resistant to AZT and ddI (Table 1). This was in contrast to

previous observations in which the 74V mutation suppressed AZT resistance when 41L was not present⁷. Addition of the NNRTI-resistance mutation 181C (41L, 74V, 181C, 215Y) produced virus cross-resistant to ddI and nevirapine, but sensitive to AZT (Table 1). Our previous studies¹⁴ indicated however, that AZT and nevirapine co-resistance could occur by *in vitro* selection for nevirapine resistance with mutant 41L/215Y. In this case, a mutation appeared in reverse transcriptase at codon 106, not 181 (Table 1).

We next conducted a series of selection experiments in cell culture, starting with the AZT and ddI co-resistant virus, HIVRTMZ (41L, 74V, 215Y), to assess the likelihood of triply resistant HIV-1 occurring. The results of these experiments are shown in Fig. 1. Passage of this mutant in nevirapine alone led to the rapid appearance of nevirapine-resistant virus (by passage 3), with a simultaneous reduction in AZT resistance (Fig. 1a). DNA sequence analysis of reverse transcriptase from this virus revealed the development of the Y181→C mutation, accounting

TABLE 1 Drug susceptibility of HIV-1 containing mutations associated with resistance to AZT, ddI, nevirapine and FTC

Virus	Genotype						Drug sensitivity (μM)			
	41	74	106	181	184	215	AZT	ddI	Nev	FTC
	M	L	V	Y	M	T				
HXB2	•	•	•	•	•	•	0.01	2.2	0.06	0.09
HIVRTMN	L	•	•	•	•	Y	0.65	2.7	0.06	0.34
HIVRTMZ	L	V	•	•	•	Y	0.33	12.5	0.09	ND
HIVRTMT	L	V	•	C	•	Y	0.07	6.3	6.7	ND
HIVRTMNp3Nev	L	•	A	•	•	Y	1.1	ND	17.8	ND
HIVRTMDR1	L	V	A	•	•	Y	0.94	13.1	16.5	0.10
HIVRTMDR2	L	V	A	•	V	Y	0.01	26.0	9.3	221

HIV-1 mutant viruses were constructed with varying combinations of known drug-resistance mutations in the virus-encoded enzyme reverse transcriptase (RT). The sensitivity of these viruses to a diverse range of RT inhibitors was determined in the HeLa CD4⁺ plaque-reduction assay. The fifty per cent inhibitory concentration (IC₅₀) was determined from plots of percentage plaque reduction against drug concentration. The virus HIVRTMNp3Nev was derived from the serial passage of HIVRTMN in escalating concentrations of nevirapine (Nev) as described¹⁴. Mutations at codons 74 (Leu to Val), 106 (Val to Ala), 181 (Tyr to Cys) and 184 (Met to Val) were introduced into the RT containing M13 clone mpRTMN (41L, 215Y)¹⁶ by site-directed mutagenesis (USB T7 gene mutagenesis kit). M13 plaques were assayed for RT activity as described²⁸ and the presence of the new mutations confirmed by DNA sequencing analysis (Sequenase). The mutated RT was then transferred into the HXB2 genetic background by recombination with the RT-deleted molecular clone pHIVARTstEII as described^{7,16}. Cell-free virus supernatant was collected from cell culture after 14–16 d and stored at –70 °C. Virus aliquots were titred and drug sensitivities determined using HeLa CD4⁺ cells²⁰. Values represent the mean of 2–4 independent determinations. ND, not determined.

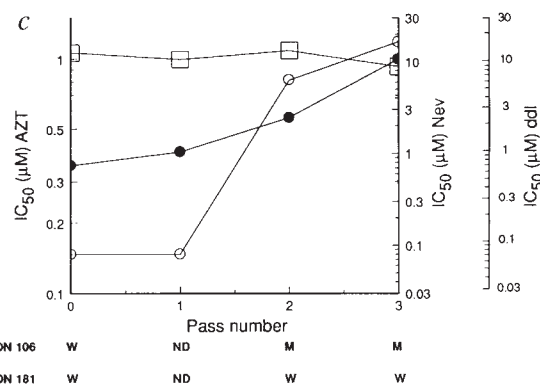
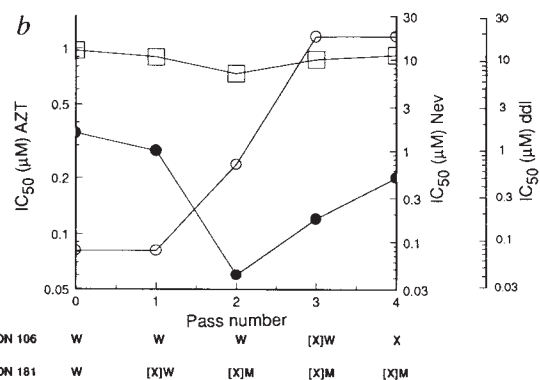
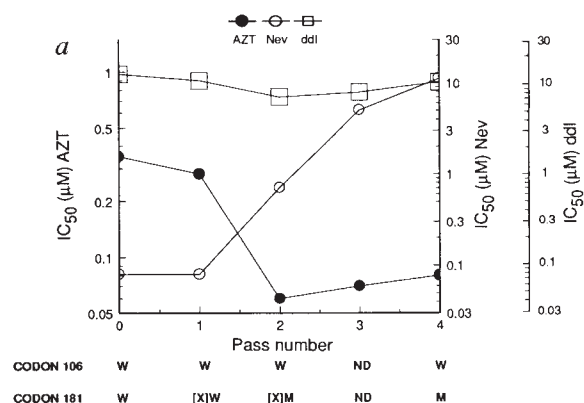
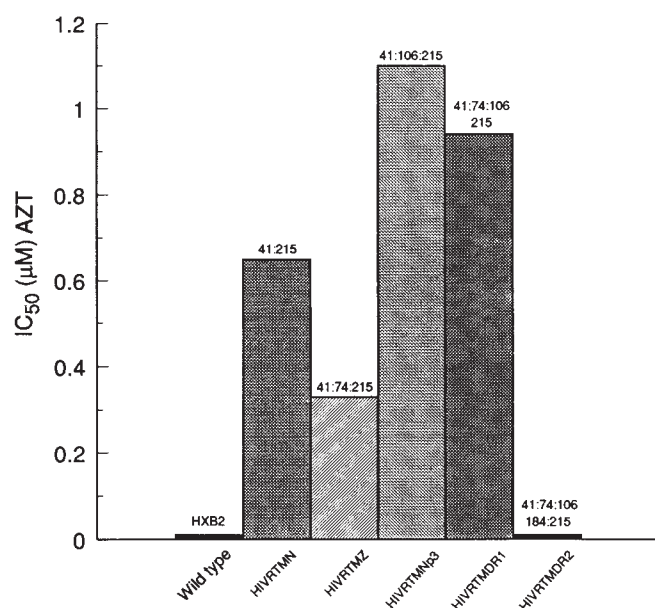


FIG. 1 *In vitro* drug sensitivity of the site-directed mutant virus HIVRTM2 passed in: a, increasing concentrations of nevirapine (Nev); b, first nevirapine, then increasing concentrations of AZT plus nevirapine; and c, ddI plus increasing concentrations of AZT and nevirapine. The presence of mutations in regions of the reverse transcriptase associated with resistance to nevirapine were determined by direct sequence analysis of PCR products obtained from DNA extracted from infected cells. METHODS. 2×10^4 p.f.u. ml^{-1} of cell-free HIVRTM2 was used to infect 2×10^6 MT-2 cells in 1 ml virus supernatant for 1 h. Infected cells were then added to 10 ml drug-containing medium (RPM1 supplemented with 10% foetal calf serum plus antibiotics). Virus was cultured with the addition of fresh drug-containing medium as required until peak cytopathic effect was reached. At this time, cell-free virus supernatant was collected and stored in 1-ml aliquots at -70°C . A 1-ml aliquot of supernatant was used to reinfect 2×10^6 MT-2 fresh cells for each successive passage. Drug concentrations used for each passage (1–4) were: a, (1) Nev 0.2 μM ; (2) Nev 0.3 μM ; (3) Nev 1.0 μM ; (4) Nev 5.0 μM ; b, (1) Nev 0.2 μM ; (2) Nev 0.3 μM ; (3) Nev 1.0 μM , AZT 0.4 μM ; (4) Nev 5.0 μM , AZT 1.0 μM ; c, (1) Nev 0.08 μM , AZT 0.15 μM , ddI 15 μM ; (2) Nev 0.3 μM , AZT 0.2 μM , ddI 5.0 μM ; (3) Nev 0.5 μM , AZT 0.5 μM , ddI 5.0 μM . It should be noted that passages 0 to 2 in selection b were obtained from selection a and then passaged in increasing concentrations of AZT and nevirapine. Virus sensitivity was determined in the HeLa CD4⁺ plaque-reduction assay as described²⁰, except that the virus supernatant was removed from the cell monolayers after a 1-h adsorption to remove carry-over drug in the infecting supernatant. Infected cell pellets were stored at -70°C from each passage and the DNA extracted using an AUTOGEN 540 DNA extractor. A subfragment of RT

was amplified by PCR using the primers A(35) and NE1(35) as described²⁹. The PCR products were directly DNA sequenced (Sequenase) using primer HIV-B²⁹ to sequence through the codon-181 region, and primer HIV-comb 5 (5'-GGG AAC TGA AAA ATA TGC-3') to sequence through the codon-106 region. DNA sequence was scored at codon 181, Tyr wild-type (W), Cys mutant (M), mixed codon of wild type and mutant but predominantly wild type ([X]W), mixed codon but predominantly mutant ([X]M). The DNA sequence at codon 106 was scored, Val wild type (W), Ala mutant (M), mixture containing equal intensity wild type and mutant sequence (X), mixture of wild type and mutant but predominantly wild type ([X]W). ND, not determined.



for the observed phenotype (Fig. 1a). But when virus from passage 2 was subsequently passaged in nevirapine plus AZT, reappearance of AZT-resistant virus was noted (Fig. 1b). Genetic analysis of the virus transcriptase suggested that this phenotype was due to the gradual induction of the V106→A mutation, with a mixture of wild-type and mutant sequences remaining at codon 181 (Fig. 1b). Finally, passage of HIVRTM2 concurrently in AZT, ddI and nevirapine led to the rapid development of nevirapine resistance and, curiously, an actual increase in AZT resistance (Fig. 1c). DNA sequencing showed that the V106→A mutation had appeared in the reverse transcriptase, while codon 181 remained wild type (Fig. 1c). As ddI resistance persisted during these experiments, selection with all three inhibitors clearly resulted in the rapid development of triply resistant HIV-1, starting with dual-resistant virus.

To confirm that the genotype seen in the third passage series (Fig. 1c) could account for the triple-resistant phenotype, we

FIG. 2 AZT sensitivity of HIV-1 containing combinations of mutations associated with multiple-drug resistance. Mutant viruses were constructed as described in Table 1 legend, and the sensitivity to AZT determined in the HeLa CD4⁺ plaque reduction assay²⁰.

TABLE 2 Viability of HIV-1 containing multiple mutations in reverse transcriptase

Virus genotype	Recombinant RT activity (%)	Virus from transfection HeLa CD4 ⁺ titre (PFU ml ⁻¹)	Second passage virus HeLa CD4 ⁺ titre (PFU ml ⁻¹)	Second passage virus C8166 titre (TCID ₅₀ ml ⁻¹)
HXB2	100	4.6 × 10 ⁴	6.2 × 10 ⁴	10 ^{4.95}
HIVRTMDR1	70	3.5 × 10 ⁴	5.3 × 10 ⁴	10 ^{4.45}
74V/103N/215Y	95	1.0 × 10 ⁵	5.0 × 10 ⁴	10 ^{4.45}
74V/103N/215Y/219Q	91	2.6 × 10 ⁵	5.4 × 10 ⁴	10 ^{4.45}

HIV-1 mutant viruses were constructed with previously reported growth-attenuating mutations in the reverse transcriptase gene²² and the viruses' ability to replicate was compared with wild-type HIV-1 (HXB2) and HIVRTMDR1 (Table 1). The bacterially expressed recombinant RT activity was determined for each mutant²⁸ and the viability of the viruses was assessed by culture and virus titration in diverse cell lines. The genotype of mutants 74V, 103N, 215Y and 74V, 103N, 215Y, 219Q was verified by direct DNA sequence analysis of PCR products obtained from MT-2 cells reinfected with these viruses. In addition, full-length active RT clones were obtained in M13 from the same material and the DNA sequence of the entire RT coding region was determined. No differences were noted between the sequence of clones obtained from the cultured viruses compared with the original M13 RT mutant clones used for transfection. HIVRTMDR1 was constructed as described in Table 1 legend. Mutants 74V, 103N, 215Y and 74V, 103N, 215Y, 219Q (MT4) were obtained by the introduction of mutations 103N and 219Q into the M13 RT clone already containing mutations 74V and 215Y (ref. 7) as described for Table 1 legend. Crude cell lysates of bacterially expressed RT were used in *in vitro* reverse transcriptase assays as described²⁸. RT activities are expressed as a percentage of wild-type (HXB2) RT activity. Virus stocks were prepared as outlined in Table 1 legend and were titrated in HeLa CD4⁺ cells. 2 × 10⁴ p.f.u. ml⁻¹ of each mutant virus stock was used to infect 2 × 10⁶ MT-2 cells as described in Fig. 1 legend. At peak cytopathic effect, cell-free virus supernatants were collected and the virus titre determined in HeLa CD4⁺ cells (p.f.u. ml⁻¹) and by end-point dilution in C8166 cells (TCID₅₀). DNA was extracted from the MT-2 cell pellet, following a second round of infection with cell-free virus. The entire RT coding region was amplified by PCR and cloned into M13 as described¹⁵. The complete nucleotide sequence of active RT clones was determined¹⁵.

added the 106A mutation by site-specific mutagenesis to the HIVRTMZ background (41L, 74V, 106A, 215Y). Sensitivity analysis of the resulting virus, HIVRTMDR1 (41L, 74V, 106A, 215Y), confirmed that it was co-resistant to ddI and nevirapine and also that the level of AZT resistance was higher than that of HIVRTMZ (similar to the passaged virus) (Table 1). This

indicated that the partial suppression of AZT resistance by 74V in HIVRTMZ did not occur when 106A was present.

Our results demonstrating the viability of a triply resistant HIV-1 were surprising in view of recent observations by Chow *et al.*²², so we constructed an HIV-1 having the same combination of mutations in its reverse transcriptase (74V, 103N, 215Y, 219Q) as were previously thought to abolish virus replication (mutant MT4). We found that recombinant reverse transcriptase containing these mutations, when expressed in *Escherichia coli*, was virtually as active as the wild-type enzyme (Table 2). In addition, virus containing this combination of mutations was viable, with similar growth properties to wild-type virus in cell culture (Table 2). The reason for these conflicting results is unclear at present, but the viable virus phenotype of the MT4 mutant has also been observed by others²⁶.

HIV-1 can develop rapid high-level resistance *in vitro* to oxathiolane-cytosine nucleosides such as 2',3'-dideoxy-3'-thiacytidine (3TC) and 2',3'-dideoxy-5-fluoro-3'-thiacytidine (FTC)^{24,25}. This resistance is due to a single mutation at codon 184 in reverse transcriptase^{24,25}. When present in an AZT-resistance background, this mutation (M184→V) can reverse AZT resistance²⁵. To determine whether the 184V mutation could also influence AZT resistance in the triple-resistant background (HIVRTMDR1), we incorporated 184V by site-directed mutagenesis into HIVRTMDR1 to give virus with the genotype 41L/74V/106A/184V/215Y (HIVRTMDR2). Sensitivity assessment of this virus (Table 1) showed that it was co-resistant to ddI, nevirapine, and FTC, but, interestingly, that it had regained wild-type sensitivity to AZT (Fig. 2). This indicated that, unlike 74V, the AZT suppressive effect of 184V was dominant over the 106A NNRTI-resistance mutation.

In conclusion, we have shown that under specific *in vitro* selection conditions, dual resistant HIV-1 can rapidly acquire co-resistance to three different reverse transcriptase inhibitors. Thus it is likely that combination therapy with multiple drugs will lead to multidrug resistance, especially in individuals who already harbour dual-resistant virus. But there are still good reasons to investigate the clinical effects of simultaneous coadministration of three reverse transcriptase inhibitors, although the specific choice of drugs will probably be critical. It will be of interest to assess synergistic combinations of inhibitors with resistance properties that restore or maintain AZT sensitivity (such as 3TC or FTC and NNRTIs), in the hope of achieving a prolonged suppression of HIV-1 replication and a more profound effect on disease.

Note added in proof: Since submission of this letter, we have learned that further sequencing of mutant MT4 reported by Chow *et al.*²² has led to the identification of inadvertent lethal but drug-irrelevant mutations in the reverse transcriptase region. This error has been acknowledged by Chow *et al.*²⁷. □

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Skeletal muscle myosin light chains are essential for physiological speeds of shortening

Susan Lowey, Guillermina S. Waller & Kathleen M. Trybus

Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, Massachusetts 02254-9110, USA

In muscle each myosin head contains a regulatory light chain (LC2) that is wrapped around the head/rod junction, and an alkali light chain that is distal to LC2 (ref. 1). The role of these light chains in vertebrate skeletal muscle myosin has remained obscure^{2,3}. Here we prepare heavy chains that are free of both light chains in order to determine by a motility assay⁴ whether the light chains are necessary for movement. We find that removal of light chains from myosin reduces the velocity of actin filaments from $8.8 \mu\text{m s}^{-1}$ to $0.8 \mu\text{m s}^{-1}$ without significantly decreasing the ATPase activity. Reconstitution of myosin with LC2 or alkali light chain increases filament velocity to intermediate rates, and readdition of both classes of light chains fully restores the original sliding velocity. We conclude that even though the light chains are not essential for enzymatic activity, light-chain/heavy-chain interactions play an important part in the conversion of chemical energy into movement.

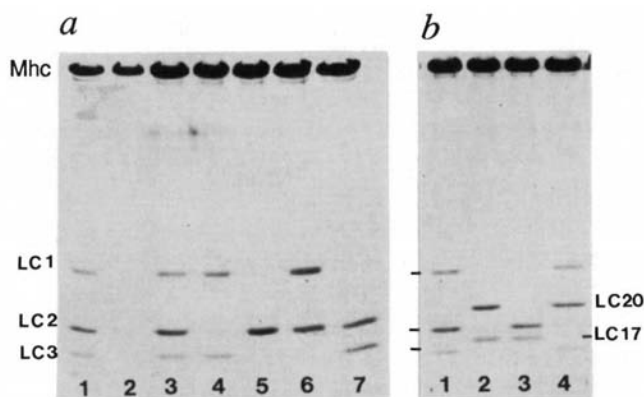


FIG. 1 Characterization of myosins reconstituted from heavy chain and different light chains by SDS-polyacrylamide gel electrophoresis. *a*, Native myosin (lane 1), isolated heavy chain (lane 2), heavy chain reconstituted with total light chains (lane 3), ALCs (lane 4), LC2 (lane 5), LC1 (lane 6), LC3 (lane 7). *b*, Heavy chain reconstituted with total skeletal light chains (lane 1), total smooth muscle light chains (lane 2), skeletal LC2 and smooth essential LC17 (lane 3), skeletal ALCs and smooth regulatory LC20 (lane 4). Mhc, myosin heavy chain.

METHODS. Chicken pectoralis myosin was incubated for 10 min at 4°C in $4.7 \text{ M NH}_4\text{Cl}$, 50 mM sodium phosphate, $\text{pH } 7.5$, 2 mM Mg-ATP , 8 mM dithiothreitol (DTT), and 1 ml (3 mg) was loaded on to a $1.6 \times 30 \text{ cm}$ Superose 6 gel filtration column (FPLC, Pharmacia) equilibrated in ammonium chloride². The protein was eluted at 1 ml min^{-1} at room temperature, and the myosin heavy chain was immediately transferred to ice. The NH_4Cl was exchanged for 0.4 M NaCl using a Sephadex G50 spun column, and the different light chains were added. To remove excess light chains, the reconstituted myosin was precipitated by overnight dialysis against 5 mM sodium phosphate, $\text{pH } 6.2$, 5 mM MgCl_2 , 1 mM DTT. After centrifugation, the pellet was washed free of light chains and resuspended in high-salt buffer (this material was analysed in the 12.5% gels shown). Skeletal muscle light chains were prepared from chicken pectoralis muscle myosin as described⁴. Smooth muscle light chains were prepared as recombinant proteins in *Escherichia coli*⁷.

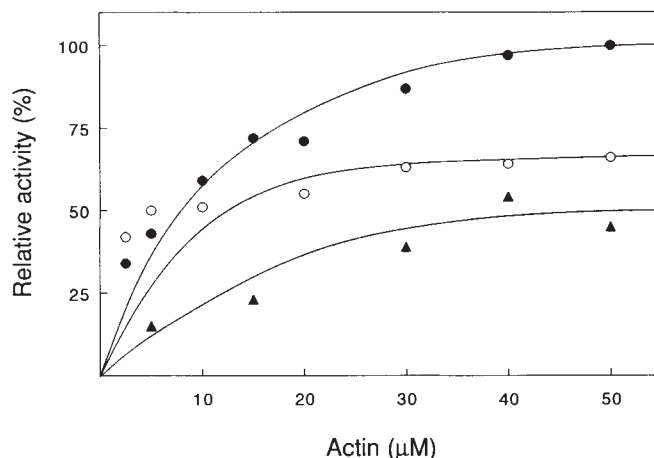


FIG. 2 Relative enzymatic activity. The actin-activated Mg^{2+} -ATPase of isolated heavy chain (▲) and LC2-myosin (○) was compared with the ATPase of myosin reconstituted with total light chains (●). The ATPase activity for myosin reconstituted with total light chains was about 80% of the value obtained for native myosin. These values represent an average of at least two experiments. Lines were drawn to favour values at higher actin concentrations as there are insufficient data to determine the Michaelis constant (K_m) for the reconstituted myosins.

METHODS. The actin-activated ATPase was determined in 25 mM KCl, 1 mM DTT, 4 mM MgCl_2 , 2.5 mM ATP, 10 mM imidazole, $\text{pH } 7.5$, at 25°C . The activity of native myosin under these conditions was about 2.5 s^{-1} . In the case of the heavy chain alone, activity was measured immediately after the G50 desalting column, because the heavy chain is unstable and tends to aggregate with time. Addition of LC2 stabilizes the heavy chain and the same ATPases were obtained after G50 column treatment as after overnight dialysis. Alkali light chains added to heavy chains gave similar ATPases to those obtained with LC2 (data not shown).

Chymotryptic digestion of skeletal muscle myosin yields a subfragment (S1) that lacks LC2 but is otherwise perfectly functional⁵. The remaining alkali light chain (ALC) can be removed by an immunoadsorbent in the presence of 4.6 M NH_4Cl (ref. 2), or by ion-exchange chromatography at 37°C (ref. 3). These mild dissociating conditions result in an S1 heavy chain which, despite being unstable, retains most of its actin-activated ATPase^{2,3}. We examined the rate of movement of fluorescently labelled actin filaments over heavy chains entirely devoid of light chains, or reconstituted with the different classes of light chain. It was necessary to use myosin rather than S1 in the motility assay because S1 supports movement of actin filaments at a much slower rate ($1\text{--}2 \mu\text{m s}^{-1}$) than myosin ($8\text{--}9 \mu\text{m s}^{-1}$ at 30°C).

No method for preparing myosin heavy chains free of light chains has been reported. We previously used antibody-affinity columns to remove LC2 from myosin⁶, but this approach is not readily applicable to ALC. We therefore modified the procedure used for exchange of ALC into S1 (ref. 5) by including gel filtration to separate light and heavy chains (Fig. 1*a*). The heavy chain isolated in the void volume (Fig. 1*a*, lane 2) is essentially free of light chain, but can rebind stoichiometric amounts of total light chains (lane 3). We also prepared myosins containing only ALCs or LC2 (Fig. 1*a*, lanes 4, 5), and the ALC isoforms LC1 (lane 6) and LC3 (lane 7).

The actin-activated Mg^{2+} -ATPase activities of the heavy chain and the various reconstituted myosins are remarkably high, considering that the myosin has been exposed to a denaturant, albeit mild, and that the isolated heavy chain is basically unstable. Myosin containing total light chains had ~80% of the maximum activity of native myosin (Fig. 2). Relative to this reconstituted myosin, the free heavy chain had ~50% activity, and myosin containing only LC2 had ~70% activity. These values are in the same range as those obtained for S1 heavy

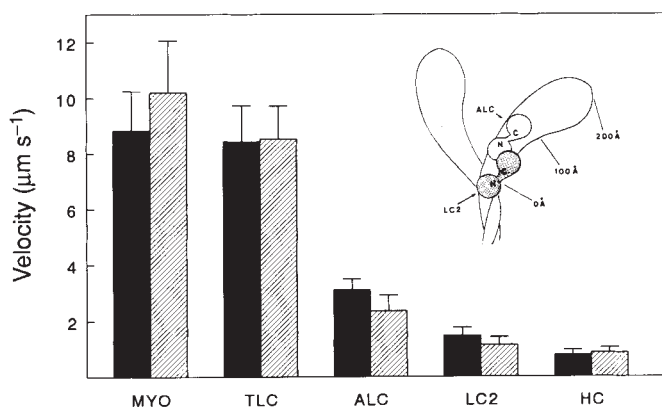


FIG. 3 Velocity of actin filaments by myosin heavy chain and reconstituted myosins. Inset: Schematic diagram of the myosin head showing the position of the light chains (N and C denote N and C termini respectively; from ref. 1). The preparation of these myosins is described in Fig. 1 legend. Solid bars indicate samples for which the myosin was adsorbed directly onto the nitrocellulose-coated surface. Hatched bars indicate that a monoclonal antibody (5C3), specific for the C terminus of the rod (ref. 8), was first adsorbed to the nitrocellulose and used to attach the myosin to the coverslip. The latter procedure was intended to minimize any unfavourable interactions between myosin and its substratum. The mean velocity ($\mu\text{m s}^{-1}$) and standard deviation for each sample are as follows (solid bars): native myosin (MYO), 8.84 ± 1.40 (74); total light chains (TLC), 8.43 ± 1.31 (118); ALC, 3.10 ± 0.39 (24); LC2, 1.46 ± 0.31 (98); heavy chains (HC), 0.78 ± 0.19 (66). Samples attached through the antibody (hatched bars) gave the same values within experimental error. When the number of filaments (shown in parentheses) exceeded 20, several independent experiments were done. ALC-myosin was also prepared by an immuno-adsorbent which specifically removed LC2 (ref. 6). This approach, which avoids ammonium chloride, gave similar results. To explore any differences between LC1 and LC3 isoforms, each was added separately along with LC2 to the heavy chain. Myosin reconstituted with LC1 moved actin somewhat more slowly, 6.49 ± 1.67 (20), than with LC3, 8.53 ± 1.95 (21). The effect of ALC isoforms on movement is discussed elsewhere¹⁸. Smooth muscle light chains, or combinations of skeletal and smooth light chains (Fig. 1b), gave velocities of 7.60 ± 1.07 (24) and 7.10 ± 0.82 (35), respectively. In all the experiments described here, 70–90% of the actin filaments moved.

METHODS. The *in vitro* motility assay was essentially as described elsewhere⁹. To remove any myosin that might bind to actin in an ATP-independent manner, samples were mixed with an equimolar concentration of actin in the presence of Mg-ATP and centrifuged. The following solutions (40 μl) were perfused through the flow cell: monomeric myosin (50 $\mu\text{g ml}^{-1}$) in 0.6 M KCl was adhered to the nitrocellulose-coated coverslip for 60 s; 0.5 mg ml^{-1} BSA in 0.6 M KCl (2 $\times$); 12 nM phalloidin rhodamine-labelled F-actin in assay buffer (25 mM KCl, 25 mM imidazole, pH 7.5, 4 mM MgCl_2 , 1 mM EGTA) was bound to myosin for 30 s (2 $\times$); 0.5% methylcellulose in assay buffer and oxygen scavengers (2 $\times$); assay buffer with additives of previous step plus 1 mM ATP (3 $\times$). Temperature was controlled at 30 °C with a heated objective. Actin movement was observed with an inverted microscope (Zeiss Axiovert 10) equipped for epifluorescence with a 100-W mercury lamp and rhodamine filter set. A 63 $\times$ Zeiss planapochromat objective, NA 1.4, an image-intensified CCD camera (Hamamatsu C2400-80), and an Argus 10-image processor (Hamamatsu) were used to record the actin images on videotape. To determine actin velocity, video images were digitized into a 480 $\times$ 512-pixel array by a video grabber card in a laboratory computer. A computer program¹⁹ takes video snapshots at intervals of 0.15–0.30 s for skeletal myosin and calculates a mean velocity for any designated actin filament. Velocities are presented as the mean and standard deviation of the mean of 10–20 filaments for each preparation.

chain² and support the earlier conclusion that the ALCs are not essential for actin binding or ATP hydrolysis.

When these myosins were tested for their ability to move actin filaments by an *in vitro* motility assay, the rate at which actin slides over the isolated heavy chain was reduced 10-fold com-

pared with native myosin or reconstituted myosin (Fig. 3). Addition of LC2 increased the rate from $0.8 \mu\text{m s}^{-1}$ to $1.5 \mu\text{m s}^{-1}$, and ALC raised the rate to $3.1 \mu\text{m s}^{-1}$, but both partially reconstituted myosins gave rise to sliding velocities considerably less than the value of $8.4 \mu\text{m s}^{-1}$ obtained for myosin reconstituted with total light chains (Fig. 3).

We also determined whether the rate of actin filament movement is affected by the type of light chain used in the reconstitution. Total smooth muscle light chain (Fig. 1b, lane 2) or combinations of skeletal and smooth light chains (lanes 3, 4) were added to the skeletal heavy chain. The velocity of actin movement over these hybrid myosins was about the same as that observed for myosin reconstituted from the homologous light chains (Fig. 3 legend). These results are in contrast to those obtained with smooth muscle heavy chain, where added skeletal LC2 completely inhibited both ATPase activity and actin movement⁷.

Slower velocities could be obtained by an unfavourable interaction between myosin and the nitrocellulose surface to which it adheres. To avoid this problem, we first adsorbed the nitrocellulose-coated coverslip with a monoclonal antibody specific for the carboxy-terminal end of the myosin rod⁸. Capturing the myosin molecule by the antibody interaction clearly did not increase the values obtained for the sliding velocities (Fig. 3). Another concern was that the low rate obtained for the heavy chain might be due to partial denaturation during its preparation for the motility assay. This point was addressed by adding light chains at various times after NH_4Cl removal: a 15–20-min delay did not affect the reversibility of the rate ($7.63 \pm 1.22 \mu\text{m s}^{-1}$), although a 50% drop in filament velocity occurred after 2 h ($4.36 \pm 0.83 \mu\text{m s}^{-1}$). Another control was to take myosin reconstituted with LC2, which had been precipitated overnight to free it of excess LC2, and measure its sliding rate before ($1.16 \pm 0.30 \mu\text{m s}^{-1}$) and after ($8.60 \pm 1.35 \mu\text{m s}^{-1}$) addition of ALCs in solution or directly in the flow chamber ($6.32 \pm 1.11 \mu\text{m s}^{-1}$). The clear reversibility of rates argues against denaturation during the preparative procedures.

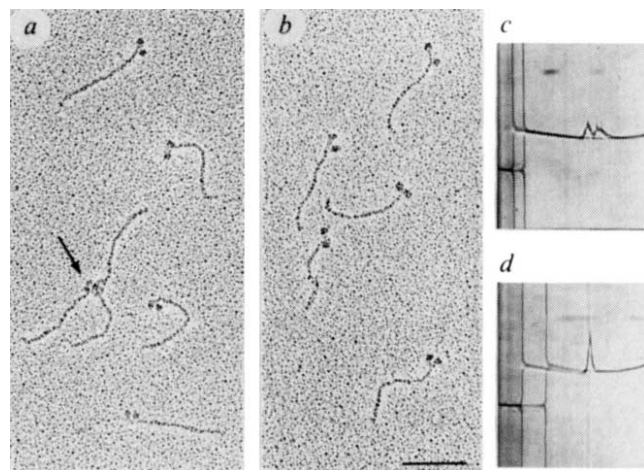


FIG. 4 Characterization of reconstituted myosins by electron microscopy and analytical ultracentrifugation. a and b, Metal-shadowed images of heavy chain reconstituted with ALC and LC2, respectively. Arrow, trimer. c and d, Sedimentation patterns of ALC-myosin and LC2-myosin, respectively. Scale bar, 100 nm.

METHODS. Reconstituted myosin was diluted to 15 $\mu\text{g ml}^{-1}$ in 0.5 M ammonium acetate, 66% glycerol, and rotary shadowed with platinum⁷. Electron micrographs were obtained on a Philips EM301 microscope operated at 60 kV. Sedimentation velocity measurements were made in a Beckman Model E analytical ultracentrifuge at 60,000 r.p.m. using 1–2 mg ml^{-1} protein.

Can the slower velocities be accounted for by a minor population of strong binding molecules, other than rigor heads (Fig. 3 legend), which impose an internal load on the remaining faster-cycling myosins? This possibility was tested using mixtures of isolated heavy chain and native chicken myosin: at a ratio of 1:1 the velocity was about half of that for myosin alone. This is a much smaller effect than for smooth muscle myosin, which slowed actin movement over skeletal myosin by as much as 90% in 1:1 mixtures⁹. It is unlikely therefore that a small population of stronger binding molecules in the reconstituted myosin inhibits actin movement.

The structural state of the different myosin species was examined by both electron microscopy and analytical centrifugation. Metal-shadowed images of heavy chain alone and to which ALCs had been added, showed dimers and trimers as well as monomers in the field, consistent with the distribution found earlier for myosin from which LC2 had been removed by a specific antibody (Fig. 4a)⁶. Unexpectedly, heavy chain reconstituted with only LC2 was remarkably monomeric (Fig. 4b). Inspection of the rotary-shadowed molecules indicated that the heads of myosins deficient in light chains were more rounded and collapsed than heads in native myosin, similar to the shorter heads measured in desensitized scallop myosin¹⁰. The sedimentation patterns, which provide a more objective sampling of the myosin population, gave similar results (Fig. 4c, d). Myosin reconstituted with ALC contained about 50% monomers, whereas myosin reconstituted with LC2 was entirely monodisperse. These data strongly suggest that the slow rate of actin movement over LC2-myosin cannot be ascribed to aggregation, particularly as ALC-myosin moved actin faster despite being more oligomeric.

Some *in situ* data on the effects of light chain removal can be compared with the *in vitro* motility studies. Partial removal of LC2 by EDTA treatment of skinned fibres markedly reduced the maximum velocity of shortening, with little effect on isometric tension^{11,12}. Unfortunately, there is no method to remove ALCs reversibly from fibres, but the application of molecular genetic techniques to *Dictyostelium* has made it possible to generate a mutant cell line deficient in ALC expression¹³. These cells failed to develop normally, and in general resembled myosin heavy-chain null mutants.

Crystallographic structures of the myosin head (S1) (ref. 14) and the regulatory domain of S1 (ref. 15 and C. Cohen, personal communication) show that the light chains are associated with a 10-nm stretch of α -helix N-terminal to the invariant proline. It has been suggested that small conformational changes in the more distal globular domain of S1 during ATP binding and hydrolysis might be amplified by virtue of tilting this extended light-chain/heavy-chain domain, so producing a working stroke of ≥ 4 nm (refs 16, 17). In such a model, removal of the light chains would result in a collapse of the now unstable single-chain α -helix, leading to a smaller working stroke or step size. This is in fact what we have observed: removal of the ALC caused a six-fold decrease in actin velocity, and removal of both classes of LCs reduced it by up to 10-fold. These results indicate that the globular region of myosin can function as an actin-activated ATPase and minimal motor, but that the region containing the light chains is important for the transduction of the energy of hydrolysis into rapid movement. □

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cGMP mobilizes intracellular Ca^{2+} in sea urchin eggs by stimulating cyclic ADP-ribose synthesis

Antony Gallione, Alison White, Nicholas Willmott, Michelle Turner, Barry V. L. Potter* & Stephen P. Watson

University Department of Pharmacology, Mansfield Road, University of Oxford, Oxford OX1 3QT, UK

* School of Pharmacy and Pharmacology, and Institute for Life Sciences, University of Bath, Claverton Down, Bath BA2 7AY, UK

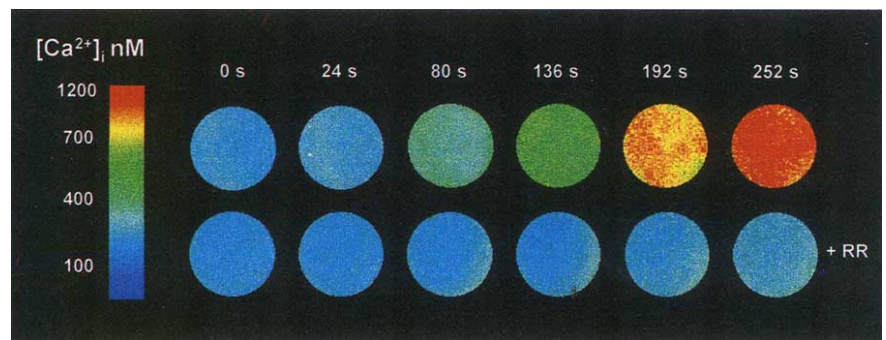
MANY hormones or neurotransmitters act at cell surface receptors to increase the intracellular free calcium concentration, triggering a wide range of cellular responses¹. As the source of this Ca^{2+} is often internal stores, additional messengers are required to convey the hormonal message from the plasma membrane. Cyclic ADP-ribose (cADPR) has been proposed as the endogenous activator of Ca^{2+} -induced Ca^{2+} release by the ryanodine receptor in sea urchin eggs and in several mammalian cell types^{2–8,22}. A second messenger role for cADPR requires that its intracellular levels be under the control of extracellular stimuli. Here we demonstrate a novel action of 3',5'-cyclic guanosine monophosphate (cGMP) in stimulating the synthesis of cADPR from $\beta\text{-NAD}^+$ by activating its synthetic enzyme ADP-ribosyl cyclase^{9–11} in sea urchin eggs and egg homogenates. We suggest that cADPR may transduce signals generated by cell surface receptors or gaseous transmitters linked to cGMP production.

Figure 1 shows that intact sea urchin eggs microinjected with the Ca^{2+} -indicator dye Fura-2 give a large Ca^{2+} signal on exposure to a membrane-permeable form of cGMP (dibutyl cGMP; 5 mM), after a latency period of ~ 80 s. This Ca^{2+} signal was blocked by prior microinjection of the egg with ruthenium red (50 μM) (Fig. 1), which blocks release from intracellular stores via ryanodine receptors and inhibits cADPR-induced Ca^{2+} release in sea urchin eggs, but does not block the response to inositol 1,4,5-trisphosphate (InsP_3)². These results suggest that the cGMP-induced calcium transient is due to calcium mobilization as a result of a ryanodine-sensitive calcium-release mechanism.

The mechanism of cGMP-induced Ca^{2+} release was analysed directly in sea urchin egg homogenates by monitoring the effects of exogenous cGMP on microsomal Ca^{2+} release using the Ca^{2+} indicator Fluo-3 (refs 2, 12). Egg homogenates sequester Ca^{2+} into vesicular stores by an ATP-dependent mechanism and release it in response to Ca^{2+} mobilizing agents¹². cGMP (100 μM) released Ca^{2+} from non-mitochondrial stores in concentrated homogenates (50% v/v) (Fig. 2a). Ca^{2+} release by cGMP was slow, taking several minutes to reach its maximum, in contrast to the rapid release by InsP_3 or cADPR¹². However, cGMP caused little release of Ca^{2+} from dilute homogenates (5% v/v) (Fig. 2a'), routinely used to characterize microsomal Ca^{2+} release^{2,12}. Supplementing dilute homogenates with a low

FIG. 1 Ruthenium red inhibits dibutyl cGMP-induced Ca^{2+} increase in sea urchin eggs. Unfertilized sea urchin eggs were microinjected with Fura-2 (final intracellular concentration $\sim 100 \mu\text{M}$) and 5 mM dibutyl cGMP was applied extracellularly to a control egg (top sequence) and to one co-injected with ruthenium red (RR) to $\sim 50 \mu\text{M}$ (bottom sequence). The time after addition of dibutyl cGMP is indicated above the egg sequences. Similar observations were made in two other experiments.

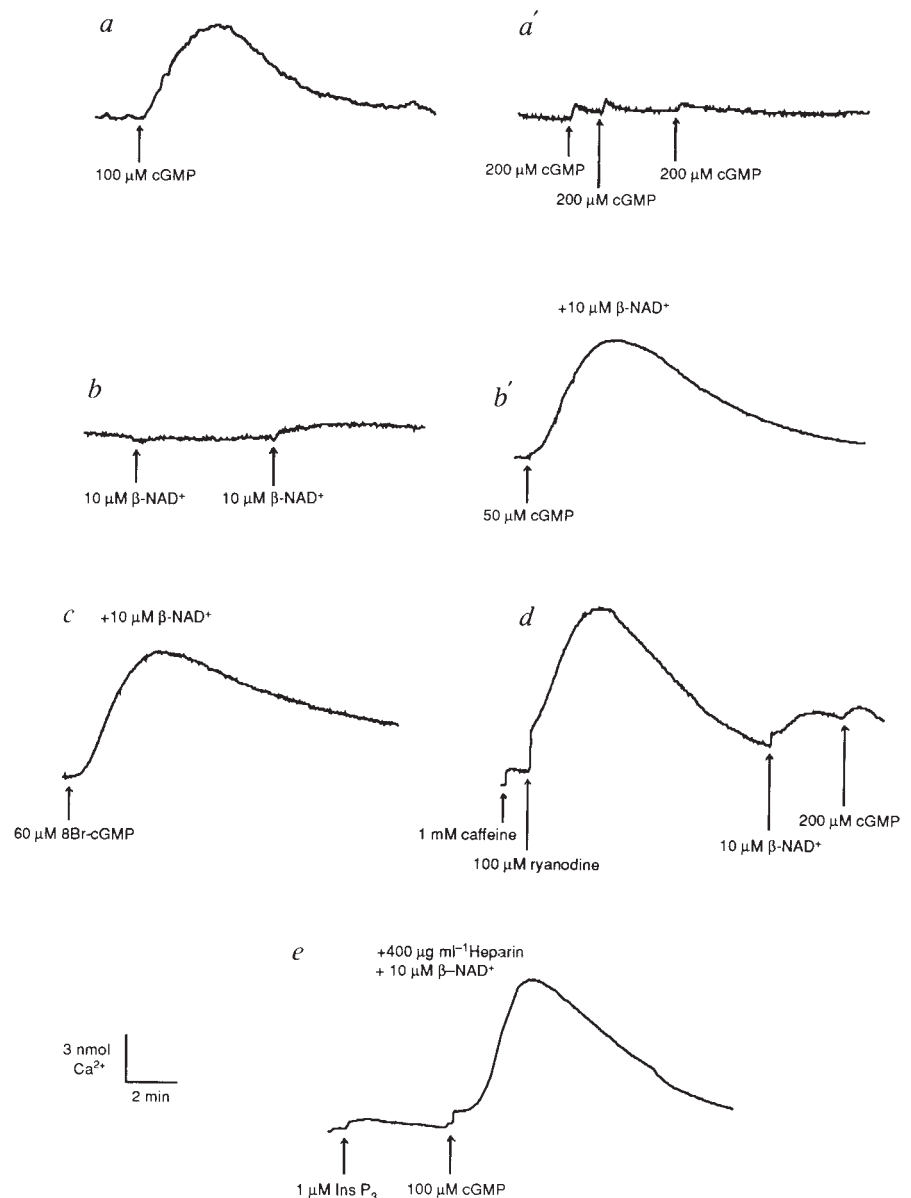
METHODS. Eggs were obtained by stimulating ovulation of female *Lytechinus pictus* (Marinus, California) with an intracoelomic injection of KCl. These were then washed twice in artificial sea water (NaCl, 435 mM; MgCl_2 , 40 mM; MgSO_4 , 15 mM; CaCl_2 , 11 mM; KCl, 10 mM; NaHCO_3 , 2.5 mM; EDTA, 1.0 mM, pH 8.0), and jelly removed by filtration through 90- μm nylon mesh. Eggs were transferred to polylysine-coated glass coverslips and microinjected with Fura-2 (10 mM in the pipette) in buffer consisting of 0.5 M KCl, 20 mM PIPES, pH 6.7, to a final cellular concentration of $\sim 100 \mu\text{M}$. In some experiments, eggs were co-injected with ruthenium red (to a final cellular concentration of $\sim 50 \mu\text{M}$) and Fura-2. Injection volumes did not exceed



1% of cell volume. All experiments were done at 22 °C. Free cytosolic Ca^{2+} concentration was determined by taking the ratio of fluorescence intensities at excitation wavelengths 340 and 380 nm, using an emission wavelength of 510 nm. Ratio images were obtained using a fluorimetric imaging system and 'lonvision' software (supplied by Improvision, University of Warwick Science Park, UK). Standard CaCl_2 solutions were used to calibrate the system and viscosity corrections were made²¹.

FIG. 2 cGMP induces Ca^{2+} release in sea urchin egg homogenate by a caffeine- and ryanodine-sensitive, but InsP_3 -insensitive mechanism. a, cGMP (100 μM) induces Ca^{2+} release in concentrated homogenate (50% v/v), but not in dilute homogenate (5% v/v) (a'). b, $\beta\text{-NAD}^+$ (10 μM) alone does not elicit an increase in Ca^{2+} , but subsequent addition of cGMP (50 μM) causes a large Ca^{2+} release in a 5% homogenate (b'). c, A slowly hydrolysable analogue of cGMP, 8-bromo-cGMP (60 μM), also causes Ca^{2+} release in a dilute ($\sim 5\%$) homogenate pretreated with $\beta\text{-NAD}^+$ (10 μM). d, Release of Ca^{2+} by caffeine (1 mM) and ryanodine (100 μM) inhibits subsequent release by cGMP in NAD^+ -pretreated homogenate. e, Heparin (400 $\mu\text{g ml}^{-1}$) blocks release by InsP_3 but not by cGMP. Each observation is representative of ≥ 4 similar experiments.

METHODS. Microsomal Ca^{2+} -loading of homogenates of eggs from *L. pictus* was achieved by incubation at 22 °C for 3 h in intracellular medium (potassium gluconate, 250 mM; N-methylglucamine, 250 mM; HEPES, 20 mM (pH 7.2); MgCl_2 , 1 mM; ATP, 1.0 mM; phosphocreatine, 10 mM; creatine phosphokinase, 10 U ml^{-1} ; oligomycin, 1 $\mu\text{g ml}^{-1}$; antimycin, 1 $\mu\text{g ml}^{-1}$; sodium azide, 1 mM; Fluo-3, 3 μM)^{2,12}. Fluorescence intensity of Fluo-3 at excitation and emission wavelengths of 490 nm and 535 nm, respectively, reflected the free $[\text{Ca}^{2+}]$ in 500- μl homogenate portions measured at 22 °C in a Perkin-Elmer LS-3 fluorimeter. Additions were made in 1–5- μl intracellular medium containing 10 μM EGTA. Calibration was by addition of CaCl_2 standard solutions. Resting $[\text{Ca}^{2+}]$ was between 100 and 150 nM. Calcium sequestration was monitored as a decrease in Fluo-3 fluorescence during microsomal loading and quantified by Ca^{2+} release in response to ionomycin (5 μM). Ryanodine and Fluo-3 were from Calbiochem; all other chemicals were from Sigma.



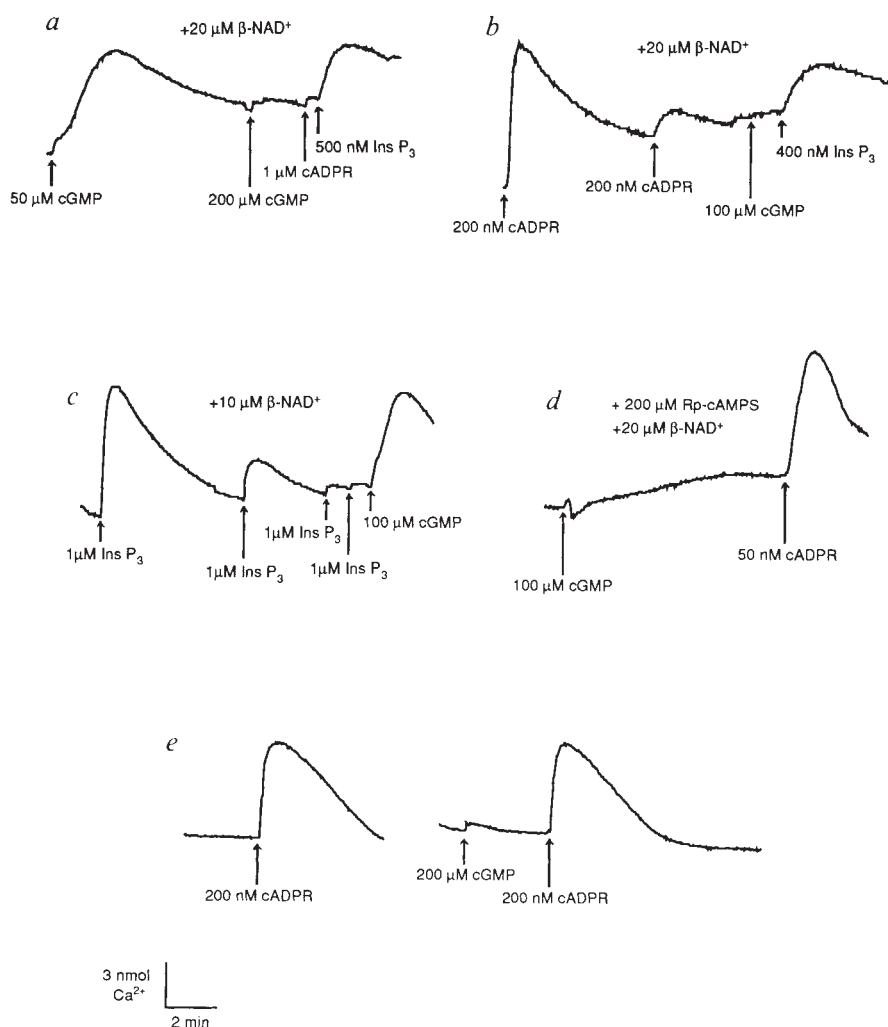
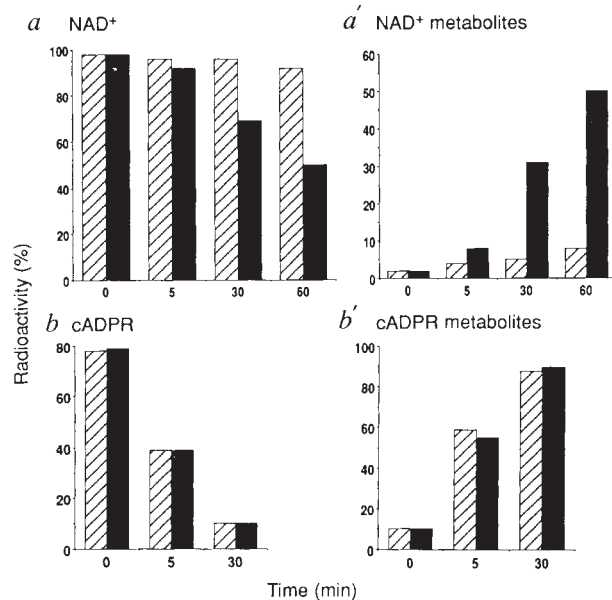


FIG. 3 cADPR- and cGMP-induced Ca^{2+} release. *a*, Release of Ca^{2+} by cGMP in the presence of $\beta\text{-NAD}^+$ desensitized the homogenate to release by subsequent additions of cGMP and cADPR but not by InsP_3 . *b*, Conversely, in cADPR-desensitized homogenate, cGMP failed to elicit a Ca^{2+} response although InsP_3 caused release. *c*, cGMP released Ca^{2+} in InsP_3 -desensitized homogenate. *d*, The inhibitor of cGMP-dependent protein kinase, Rp-cAMPS (200 μM), inhibited Ca^{2+} release by cGMP but not by cADPR. *e*, cGMP in the absence of $\beta\text{-NAD}^+$ had no effect on cADPR-induced Ca^{2+} release. Each observation is representative of ≥ 4 experiments. For experimental conditions see Fig. 2 legend.

FIG. 4 cGMP-stimulated $\beta\text{-NAD}^+$ metabolism. *a*, $[\beta\text{-}^{14}\text{C}]\text{NAD}^+$ (10 μM) metabolism in egg homogenates in the presence (filled bars) or absence (hatched bars) of cGMP (100 μM); *a'*, the majority ($>95\%$) of the radioactivity in control and cGMP-stimulated homogenates appeared as a single peak, tentatively identified as ADPR. Similar results were seen in two other experiments. *b*, $[\text{H}]\text{cADPR}$ (40 nM) metabolism in egg homogenates in the presence (filled bars) or absence (hatched bars) of cGMP (100 μM); *b'*, appearance with time of $[\text{H}]\text{cADPR}$ metabolites, 95% of which co-migrated with ADPR the major $[\beta\text{-}^{14}\text{C}]\text{NAD}^+$ metabolite. Similar results were seen in two other experiments.

METHODS. Egg homogenates (5% v/v intracellular medium) were incubated with cGMP as described in Fig. 2 legend. $[\beta\text{-}^{14}\text{C}]\text{NAD}^+$ was added to homogenates (50 μl) and incubated for 60 min at 22 $^{\circ}\text{C}$. Reactions were stopped by adding an equal volume of ethanol (100%) and then incubated on ice to precipitate protein. Aliquots were centrifuged for 10 min at 13,000g and the supernatant removed for analysis by TLC. Two TLC systems were used to analyse the reaction products. Merck silica gel $\text{NH}_2\text{F}_{2545}$ HP TLC plates (10 cm $\times$ 10 cm) were developed using $\text{H}_2\text{O}/\text{C}_2\text{H}_5\text{OH}/\text{NaCl}$ (30%/70%/0.2 M) as running solvent; 0.5 cm $\times$ 1 cm squares from the origin to the solvent front were then scraped and counted. ADPR, ADP, AMP, cADPR and $\beta\text{-NAD}^+$ ran with approximate R_f values of 0.1, 0.26, 0.47, 0.55 and 0.7 respectively. A TLC system using Merck PEI-cellulose TLC plates (10 cm $\times$ 20 cm) was also developed using NH_4HCO_3 as solvent, which gave essentially the same results, although the individual R_f values differed (A.W. et al., manuscript in preparation). $[2,5,8\text{-}^3\text{H}]\text{cADPR}$ (specific activity, 59 Ci mmol $^{-1}$) and nicotinamide $[\text{H}]\text{adenine dinucleotide}$ 276 mCi mmol $^{-1}$ were from Amersham International, UK.



concentration of β -NAD⁺ (10 μ M), which itself had a negligible Ca²⁺-releasing effect (Fig. 2b), restored the ability of cGMP (50 μ M) to induce a large Ca²⁺ release (Fig. 2b'). In contrast, α -NAD⁺ (10–200 μ M) was ineffective in potentiating cGMP-induced Ca²⁺ release (data not shown). Ca²⁺ release by cGMP in the presence of β -NAD⁺ occurred with a latency of about 30 s, suggesting that the effect of cGMP was indirect. The slowly hydrolysed analogue of cGMP, 8-Br-cGMP (60 μ M) also caused Ca²⁺ release in dilute microsomes pretreated with β -NAD⁺ (Fig. 2c), as did the non-hydrolysable thioanalogue of cGMP, Sp-cGMPS^{13–15} (data not shown), indicating that cGMP hydrolysis was not necessary. Other nucleotides, 2',3'-cGMP, 5'-GMP, GTP, and cAMP (all at 100 μ M), which unlike cGMP are unable to activate sea urchin eggs upon microinjection¹⁵, did not induce Ca²⁺ release, either alone or in the presence of β -NAD (data not shown). Prior release of Ca²⁺ by ryanodine and caffeine inhibited further release by cGMP (Fig. 2d). In contrast, the InsP₃ antagonist heparin blocked release by InsP₃, but not by cGMP (Fig. 2e). These data indicate that cGMP may cause release by means of a ryanodine-sensitive but InsP₃-insensitive mechanism.

cADPR, like cGMP, releases Ca²⁺ by a mechanism sensitive to ryanodine and ruthenium red². We examined the possibility that cGMP causes Ca²⁺ release by activating the same Ca²⁺ release mechanism as cADPR. Release of Ca²⁺ by cGMP in the presence of low concentrations of β -NAD⁺ desensitized the homogenates to release by cADPR but not by InsP₃ (Fig. 3a). In cADPR-desensitized homogenates, cGMP failed to release Ca²⁺, although InsP₃ was still effective (Fig. 3b). This suggested that cGMP releases Ca²⁺ by the cADPR-sensitive mechanism. That cGMP does not act through the InsP₃ receptor was confirmed by the finding that cGMP could still release Ca²⁺ in homogenates desensitized to InsP₃ (Fig. 3c). The mechanism of this indirect action of cGMP was revealed by experiments using adenosine 3',5'-cyclic monophosphorothioate (Rp-cAMPS), which inhibits cGMP-dependent protein kinase¹⁶. Rp-cAMPS blocked Ca²⁺ release by cGMP, although this agent did not block release by cADPR (Fig. 3d). Similar results were seen with the nonspecific protein kinase inhibitor staurosporine (10 μ M) (not shown). These data indicate that cGMP-induced Ca²⁺ release is mediated by activation of a kinase. cGMP had no effect on Ca²⁺ release induced by cADPR (10–200 nM) (Fig. 3e).

These data indicate that cGMP could be stimulating ADP-ribosyl cyclase, the enzyme that converts β -NAD⁺ to cADPR^{9–11}. This was tested directly by examining the effects of cGMP on β -NAD⁺ metabolism and cADPR synthesis. Two thin-layer chromatography (TLC) systems were developed to monitor the activity of the ADP-ribosyl cyclase pathway in sea urchin egg homogenates (Fig. 4). In control homogenates there was a small (~5%) decrease in [β -¹⁴C]NAD⁺ over 60 min, whereas treatment with cGMP increased this value to ~50% (Fig. 4a). In both cases the decrease in radioactivity in the [β -¹⁴C]NAD⁺ fraction was predominantly (>95%) recovered in a single peak (Fig. 4a') which co-eluted with the principal metabolite of [³H]cADPR (Fig. 4b). This metabolite has been tentatively identified by co-chromatography on two TLC systems as ADP-ribose¹⁰, which has no Ca²⁺-mobilizing activity^{8,10,12}. In contrast, cGMP had no effect on the rapid metabolism of [³H]cADPR (Fig. 4b'). These data suggest that cGMP enhances the metabolism of β -NAD⁺ to cADPR and that this underlies cGMP-induced Ca²⁺ release.

We have shown that cADPR synthesis from β -NAD⁺ can be enhanced by cGMP in sea urchin eggs and egg homogenates, leading to a release of stored calcium through a ryanodine-sensitive release channel. cADPR may therefore function as a calcium-mobilizing intracellular messenger for ligands activating guanylyl cyclases, including the gaseous transmitter nitric oxide. The latency, duration and magnitude of the cGMP-induced calcium transient in the sea urchin egg uniquely resembles the pat-

tern of Ca²⁺ response seen at fertilization^{15,17–19}, suggesting that cGMP may have a key role in this process. The ADP-ribosyl cyclase signalling pathway that is described here may also be an important signal transduction system, working either alone or with InsP₃ to produce the complex patterns of Ca²⁺ signals seen in mammalian cells^{1,20}. □

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Valosin-containing protein, VCP, is a ubiquitous clathrin-binding protein

Irene T. Pleasure, Mark M. Black* & James H. Keen†

Department of Pharmacology and the Jefferson Cancer Institute, Thomas Jefferson University, 233 South 10th Street, Philadelphia, Pennsylvania 19107, USA

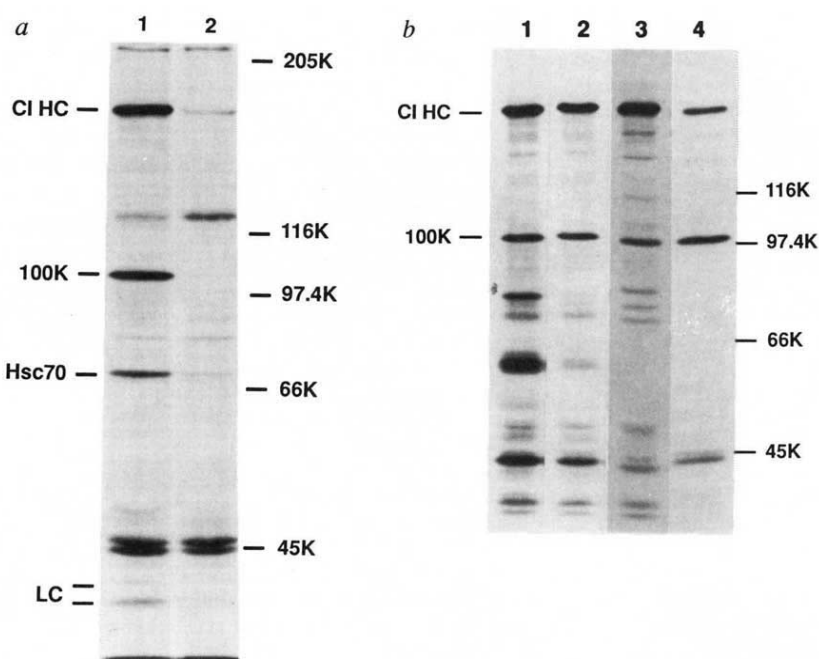
* Department of Anatomy and Cell Biology, Temple University School of Medicine, 3420 North Broad Street, Philadelphia, Pennsylvania 19140, USA

CLATHRIN is the structural protein of coated membranes involved in receptor-mediated endocytosis and aspects of Golgi sorting in eukaryotic cells. We have now detected a stoichiometric complex of clathrin with a novel protein of *M_r* ~100,000 (100K) in lysates of different mammalian cells. Formation of the complex, which also includes the 70K heat-shock protein Hsc70, occurs within 15 min of synthesis. The 100K protein has been identified as valosin-containing protein (VCP; ref. 1), an early substrate for tyrosine phosphorylation on T-cell receptor activation². Further, VCP is the mammalian homologue of yeast Cdc48p (ref. 3) and is a member of a larger gene family that includes putative ATP-binding proteins involved in vesicle transport and fusion^{4,5}, 26S proteasome function⁶, regulation of the expression of human immunodeficiency virus^{7,8}, and assembly of peroxisomes⁹. The association with clathrin and the morphological and catalytic similarity to the chaperonin proteins indicate that VCP may modulate protein-protein interactions in membrane transport processes.

† To whom correspondence should be addressed.

FIG. 1 A novel 100K protein is specifically complexed with clathrin in mammalian cell lysates. *a*, A metabolically labelled rat sympathetic neuronal extract containing soluble clathrin was challenged with anticlathrin antiserum under non-denaturing conditions. The immunoprecipitate contained stoichiometric amounts of a 100K band together with clathrin heavy chains (CI HC) and light chains (LC) and Hsc70 (lane 1). Preadsorption of the antibody with purified clathrin blocked the appearance of these bands (lane 2). Release of assembled clathrin by treatment with 0.5 M Tris-HCl, 1% Triton X-100, pH 7.2, followed by immunoprecipitation, also revealed the 100K band but at reduced levels, partly at least as a result of the high salt concentration (data not shown). *b*, Stoichiometric amounts of a 100K band are also present in anti-clathrin immunoprecipitates from extracts of metabolically labelled U251 human glioma (lane 1), RD human rhabdomyosarcoma (lane 2), primary rat cardiac muscle (lane 3) and BALB/c 3T3 mouse fibroblast (lane 4) cells; in each case, preadsorption of the antibody with clathrin blocked the appearance of clathrin and the 100K band (data not shown), as in *a*.

METHODS. Rat sympathetic neurons¹¹ were labelled for 18 h with [³⁵S]Translabel (³⁵S-L-methionine and ³⁵S-L-cysteine; ICN Flow) and lysed in buffer A (0.1 M NaMES (sodium 2-[N-morpholino]ethane sulphonate), pH 6.5, 1.0 mM EGTA, 0.5 mM EGTA, 0.5 mM MgCl₂, 0.02% sodium azide) supplemented with 0.2% Triton X100, 0.5% Trasylol and 10 µg ml⁻¹ each of leupeptin, chymostatin and antipain. Lysates were incubated on ice for 10 min at 4 °C, cleared in a



Beckman TL100.2 rotor at 75,000 r.p.m. for 10 min at 4 °C and then immunoprecipitated using anti-clathrin (LCb) antiserum¹¹. Protein A-Sepharose-bound immune complexes were recovered¹⁷ and analysed by SDS-PAGE (5–8.5% gel) and fluorography.

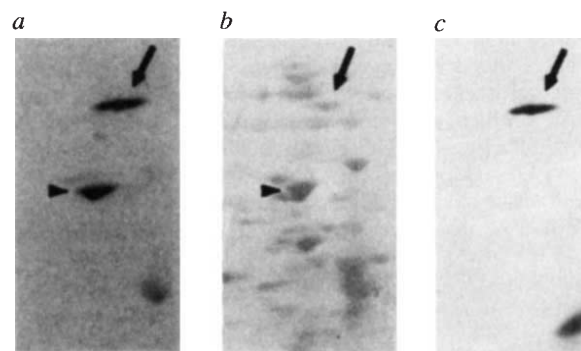
Primary rat sympathetic neurons were lysed with detergent under conditions that release unassembled clathrin in a soluble form while retaining assembled clathrin¹⁰. Immunoprecipitation under non-denaturing conditions with a polyclonal anti-clathrin light-chain b antiserum recognizing intact clathrin triskelia revealed stoichiometric amounts of a novel ~100K polypeptide, as well as clathrin heavy and light chains and a protein previously identified¹¹ as the 70K heat-shock cognate protein Hsc70 (Fig. 1, lane 1). Preadsorption of the antiserum with purified bovine brain clathrin blocked the appearance of the 100K protein as well as clathrin and Hsc70, although minor nonspecific bands persisted (Fig. 1, lane 2). A ~100K species was also observed in comparable amounts in anti-clathrin immunoprecipitates from a variety of primary and established cell lines (Fig.

1b), as well as Jurkat T cells and human natural killer cells (data not shown). On two-dimensional polyacrylamide gel electrophoresis (PAGE) of the rat neuronal immunoprecipitate, the 100K protein focused as a slightly elongated but discrete spot with pI ~5.5 (Fig. 2a). This neuronal 100K protein comigrated with a readily detectable protein in homogenates of human U251 (Fig. 2b), neuronal and BALB/c 3T3 cells (data not shown). Comigration was similar when U251 or BALB/c 3T3 cells were the source of the radiolabelled immunoprecipitates. These results indicate that the 100K protein is extremely similar or identical in these cell types, and that it is a ubiquitous clathrin-binding protein.

Direct microsequencing of the human U251 protein from two-dimensional gels was unproductive, suggesting a blocked N

FIG. 2 The 100K band in anti-clathrin immunoprecipitates is valosin-containing protein, or VCP. Two-dimensional gel electrophoresis and immunoblot analysis of radiolabelled neuronal immunoprecipitates mixed with unlabelled U251 whole-cell homogenates reveal comigration of the 100K immunoprecipitate band with a prominent cell protein identified as VCP. *a*, Autoradiograph of an anti-clathrin immunoprecipitate from radiolabelled rat neurons showing the region of the gel containing the 100K (arrowed; pI ~5.5) and Hsc70 (arrowhead; pI ~5.8) proteins. *b*, Corresponding region of an identical gel transferred to nitrocellulose and stained with Coomassie blue. The protein spots that comigrate with the 100K (arrowed) and Hsc70 (arrowhead) proteins found in the immunoprecipitate are indicated. *c*, After autoradiography, the blot shown in *a* was probed with an anti-VCP antiserum. The arrow indicates the band recognized by the antiserum, which comigrates precisely with the 100K band found in *a* and *b*.

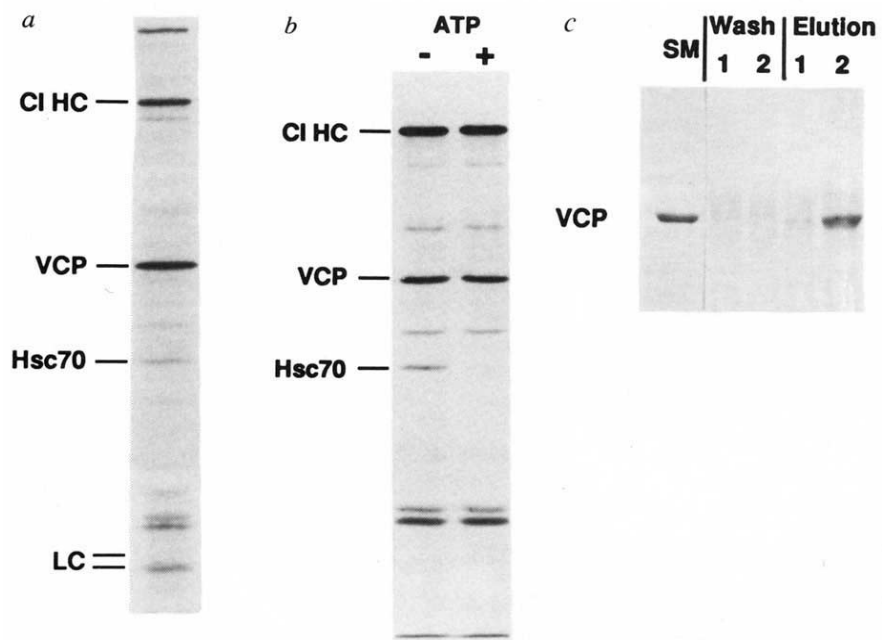
METHODS. A confluent 35-mm dish of U251 cells was lysed in 1% SDS with 5% β-mercaptoethanol, boiled and cleared by centrifugation at 14,000g for 10 min. The resultant extract was combined with an anti-clathrin immunoprecipitate from radioactively labelled rat sympathetic neurons and subjected to two-dimensional gel electrophoresis as previously described¹⁸ except that the Ampholine (Pharmacia) mixture was 0.5% pH 3.5–10 and 2.25% each of pH 6–8 and pH 5–7; an 8.5%



resolving gel was used for the second-dimension separation. The proteins were transferred to nitrocellulose and one blot was exposed to Kodak X-Omat film (*a*) followed by immunoblotting with an anti-VCP peptide antiserum (to residues 493–517; ref. 1; generously provided by K. Koller, Genentech) using a peroxidase-anti-peroxidase detection system (*c*). The other blot was stained with Coomassie blue (*b*).

FIG. 3 VCP and Hsc70 bind independently to clathrin within 15 min of synthesis in neurons. *a*, Anti-clathrin immunoprecipitation of cells labelled for 15 min demonstrates the presence of VCP and Hsc70. *b*, Addition of ATP to the immunoprecipitate releases Hsc70 but has no effect on retention of VCP. *c*, Purified VCP (starting material, SM) binds to immobilized clathrin and is released by high salt.

METHODS. *a*, Neurons were methionine-starved for 30 min, incubated with [³⁵S]Translabel for 15 min and processed as described for Fig. 1. *b*, Neurons were labelled and bead-bound immune complexes were prepared as for Fig. 1. After washing three times in the reaction buffer, components of the ATP-regenerating system (0.5 mM EDTA, 0.5 mM dithiothreitol (DTT), 1.5 mM MgCl₂, 28 U ml⁻¹ creatine phosphokinase, 15 mM creatine phosphate) were added and aliquots of the beads were incubated for 15 min at room temperature in the presence (+) or absence (-) of 1 mM ATP. Beads were then washed twice in RIPA buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% Triton X100, 0.1% SDS, 0.5% Trasylol and 10 mg ml⁻¹ each of leupeptin, chymostatin, antipain) and analysed by SDS-PAGE (5–8.5% gradient) as described for Fig. 1. *c*, Purified VCP (7.2 µg) was bound to 0.3 ml of a clathrin-Sepharose¹⁹ column (1.45 mg ml⁻¹) in buffer A with 10% glycerol for 30 min at 4 °C. The beads were washed twice with 0.6 ml of buffer A with 10% glycerol and then eluted with 2 × 0.3 ml 0.5 M Tris-HCl, pH 7.0, and analysed by 7.5% SDS-PAGE. VCP was purified from a 15S pellet of a bovine brain high-speed extract by resuspension in



25 mM Tris-HCl, 0.1 mM DTT, pH 7.0, and application to a pre-equilibrated DE-52 column (Whatman). After washing, VCP was eluted with buffer supplemented with 0.3 M KCl. 15S VCP was isolated by centrifugation, resuspended in 25 mM Tris-HCl, 150 mM KCl, 0.1 mM DTT, pH 7.0, and applied to a 5–20% sucrose gradient. 15S VCP was again recovered and its identity confirmed by SDS-PAGE and immunoblotting.

terminus. Sequences of two tryptic peptides obtained after digestion, elution and purification by high-performance liquid chromatography revealed a single sequence with near identity to a porcine protein termed valosin-containing protein or VCP¹. Specifically, two tryptic peptides were essentially identical to residues 27–41 (IVDEAINEDNSVV[X]L) and 233–238 (IGVKPP) of porcine VCP. Finally, immunoblotting using an anti-VCP peptide antiserum of a sample containing both the radiolabelled immunoprecipitate and carrier U251 cell lysate revealed a spot (Fig. 2c) that comigrated precisely with the immunoprecipitated 100K protein on the autoradiograph (Fig. 2a).

Based on the methionine and cysteine content of the proteins, approximately two molecules of VCP are bound per clathrin triskelion in the immunoprecipitate. Lysis and immunoprecipitation in the presence of glutathione (5 mM) did not diminish recovery of VCP (data not shown), indicating that nonspecific disulphide-dependent aggregation reported for the yeast VCP homologue³ is not responsible for its presence in the immunoprecipitate. VCP was largely stripped from the immunoprecipitate by washing with 2 M urea or high salt, however, accounting for its absence earlier¹¹ and suggesting that it may be bound in a transient form in cells.

In these studies cells were used that had been labelled essentially to steady state. But VCP was also readily detectable in anti-clathrin immunoprecipitates of neurons labelled for only 15 min in culture (Fig. 3a). As the clathrin complexes isolated by immunoprecipitation contain stoichiometric amounts of Hsc70 (ref. 11), a molecular chaperone that interacts with various peptides and proteins^{12,13}, we determined whether the presence of VCP in the immunoprecipitate was a result of direct binding to clathrin or to Hsc70. Addition of ATP, previously shown to release Hsc70 quantitatively¹¹, did not affect recovery of VCP (Fig. 3b). Finally, purified VCP bound directly to immobilized clathrin and was quantitatively released by high salt (Fig.

3c). These results demonstrate that VCP and Hsc70 bind independently to clathrin.

VCP has been identified as an early substrate for tyrosine phosphorylation following T-cell receptor activation². Although it is plausible that VCP-clathrin interactions are involved in signal transduction at the plasma membrane, VCP may also have other or more general activities, as only a small fraction of the protein is found in complex with clathrin. Structural studies of the *Xenopus* homologue of VCP reveal a single or stacked hexameric ring structure sedimenting at 15S (refs 14, 15), and our preliminary results indicate that mammalian VCP shares these properties. In view of the similarity of these structural and enzymatic properties to those of the GroEL class of chaperonins¹⁶, it is possible that VCP regulates protein interactions through a novel chaperone-like activity. VCP is also 70% identical to the yeast cell division cycle Cdc48 protein, and both are close relatives within a multigene family that is characterized by having one or two copies of a highly conserved ATP-binding module spanning 200 amino acids^{3,14}. Two members of this family, the N-ethylmaleimide-sensitive fusion protein NSF⁴ and its yeast homologue Sec18p (ref. 5), are required for intra-Golgi transport and targeting to the plasma membrane, and are thought to function by modulating protein-protein interactions in an ATP-dependent manner, leading to membrane fusion. Other members of this family include the S4 protein of the 26S proteasome⁶, a complex that undergoes ATP-dependent assembly, and the transcription factors, TBP-1 (ref. 7) and MSS1 (ref. 8) which bind to tat or affect tat-mediated expression of human immunodeficiency virus. The demonstration that VCP specifically and stoichiometrically binds to clathrin, its homology to proteins involved in membrane transport, and its structural and catalytic similarity to GroEL chaperonins, suggest that VCP functions in a subset of clathrin-mediated membrane transfer reactions through ATP-dependent modulation of protein-protein interactions. □

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Interaction between transcription factors Sp1 and YY1

Edward Seto*, Brian Lewis† & Thomas Shenk†

* Center for Molecular Medicine/Institute of Biotechnology, Department of Cellular & Structural Biology, University of Texas Health Science Center, San Antonio, Texas 78245-3207, USA

† Howard Hughes Medical Institute, Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544-1014, USA

A BASAL level of transcription is usually observed when all but a small region of DNA has been deleted from a eukaryotic gene promoter. These promoter elements, which are necessary and sufficient for specific transcription initiation, are referred to as minimal or core promoter elements. One element that is commonly present in a core promoter is the initiator. It has been demonstrated that the presence of Sp1 binding sites can greatly enhance the level of transcription initiation at initiator elements^{1–4}. A binding site for the YY1 transcription factor, located at the initiation site of the adeno-associated virus P5 promoter, functions as an initiator element; a synergistic enhancement of its activity is observed *in vitro* when upstream Sp1 binding sites are present³. Here we report that this synergistic activation probably occurs through protein-protein interactions.

Earlier work demonstrating the response of initiator elements to Sp1 used *in vitro* transcription extracts^{1–4}. We investigated if the interaction could be detected within cells and whether it influenced the response of a promoter to a transcriptional activating protein (Fig. 1). The presence of multiple Sp1 sites upstream of the P5 initiator, which binds YY1^{3,5}, increased chloramphenicol acetyl transferase (CAT) activity within transfected cells by a factor of six relative to constructs containing Sp1 or YY1 binding sites alone. Further, although the P5 initiator was not activated by adenovirus E1A proteins, a reporter with both the P5 initiator and Sp1 binding sites was induced by a factor of about seven in the presence of 12S but not 13S E1A protein. Replacement of Sp1 binding sites with the P5 TATA motif generated a promoter with similar basal activity to the construct with Sp1 sites, but it was not activated by E1A proteins. Thus, Sp1 sites can substantially increase the basal activity of the P5 initiator in transfected cells and, whereas neither element alone is responsive, the combination of Sp1 and P5 initiator sites is induced by a transcriptional activator.

On the basis of these data, it is reasonable to postulate that Sp1 and YY1 can selectively interact and work synergistically in combination, possibly by forming a physical complex. As a

test of this hypothesis, we constructed a fusion protein between YY1 and the activation domain of the herpes simplex virus VP16 and investigated whether it could be recruited to a promoter by Sp1 (illustrated in Fig. 2a) within *Drosophila melanogaster* Schneider-2 cells, which are devoid of Sp1⁶ and YY1³ activities. Figure 2b shows that the reporter was slightly activated by Sp1 but not by YY1/VP16. But transcription was activated by >200-fold when both Sp1 and YY1/VP16 were present. The inability of a heterologous fusion protein LAP/VP16⁷ to synergize with Sp1, and the inability of AP-2 (which also binds to the segment with Sp1 sites in the reporter⁸) to cooperate with YY1/VP16, indicated that the intracellular assay detected a specific Sp1–YY1 interaction. The actin 5C promoter of the *Drosophila* expression plasmid used to control production of Sp1 was not transactivated by YY1/VP16 in Schneider cells (data not shown), thus ruling out the explanation that YY1/VP16 increased the amount of Sp1 expressed in the transfected cells. To confirm further the specificity of the interaction, we performed competition experiments (Fig. 2c). The reporter and plasmids expressing Sp1 and YY1/VP16 were transfected into cells together with an additional plasmid which expressed the native YY1 protein. CAT induction was inhibited by YY1 in a dose-dependent fashion, presumably by competing with YY1/VP16 for binding to Sp1. To rule out a promoter-specific effect, a second reporter plasmid⁹ that contained two Sp1 binding sites¹⁰ was tested (Fig. 2d). Sp1 plus YY1/VP16, but not ATF2/VP16, induced an ~150-fold increase in CAT activity. In summary, our data suggest that Sp1 can recruit YY1 to the promoter in the absence of a YY1 binding site as a result of a protein-protein interaction. If YY1 contains an attached activation domain, as is the case for YY1/VP16, it can activate transcription. But this does not imply that YY1 normally activates transcription in the absence of its binding sites.

To obtain biochemical evidence that Sp1 and YY1 interact, we used a co-immunoprecipitation assay^{11,12} (Fig. 3a). ³²P-labelled DNA containing Sp1 binding sites was incubated with purified

	Relative CAT activity		
	Basal	12S E1A	13S E1A
pSp1nrcAT	1	0.5 ± 0.3	1.1 ± 0.1
pSp1CAT	0.9 ± 0.2	1.2 ± 0.3	0.8 ± 0.4
pSp1/P5nrcAT	6.3 ± 0.8	6.9 ± 1.4	41.7 ± 3.5
pP5TATA/P5nrcAT	7.7 ± 1.1	8.1 ± 1.4	7.1 ± 1.1

FIG. 1 The adeno-associated virus P5 initiator cooperates with Sp1 *in vivo*. P5 initiator function in transfected HeLa cells was compared in the presence or absence of adenovirus E1A proteins.

METHODS. pSp1nrcAT, pSp1CAT, pSp1/P5nrcAT and pP5TATA/P5nrcAT were constructed by inserting the CAT gene downstream of the transcription initiation sites in plasmids pP5 + 1, pSp1, pSp1/P5 + 1 and pP5TATA/P5 + 1 (ref. 3), respectively. 12S and 13S E1A were expressed from plasmids pCMV12SE1A and pCMV13SE1A¹⁶, respectively. Transfection was done using the calcium phosphate method¹⁷ and contained 5 µg reporter plasmid along with 5 µg of either pCMV12S, pCMV13S, or the parent vector pCMV. Forty-eight hours after transfection, cells were collected and CAT activity was determined in 3-h reactions. The extent of acetylation in various reactions was determined relative to that for pSp1nrcAT, and results are presented as the mean ± s.d. of three independent transfections.

Sp1 and YY1. The DNA was specifically co-immunoprecipitated with a YY1-specific antibody in a YY1 dose-dependent manner. In the absence of Sp1, no DNA was co-immunoprecipitated with YY1.

Finally, we fused the YY1 complementary DNA to the glutathione-*S*-transferase (GST) gene, and tested its ability to bind Sp1 (Fig. 3*b*). Sp1 was captured by the fusion protein, but not

by the GST polypeptide alone. Analysis of YY1 segments fused to GST indicated that the interaction with Sp1 occurred through YY1 residues 201–331.

Sp1 forms homomultimeric complexes^{13,14}, and it can bind to the bovine papillomavirus E2 protein¹² and the *Drosophila* TAF110 protein¹⁵. YY1 is the first human cellular DNA-binding transcription factor shown to bind Sp1 specifically. The inter-

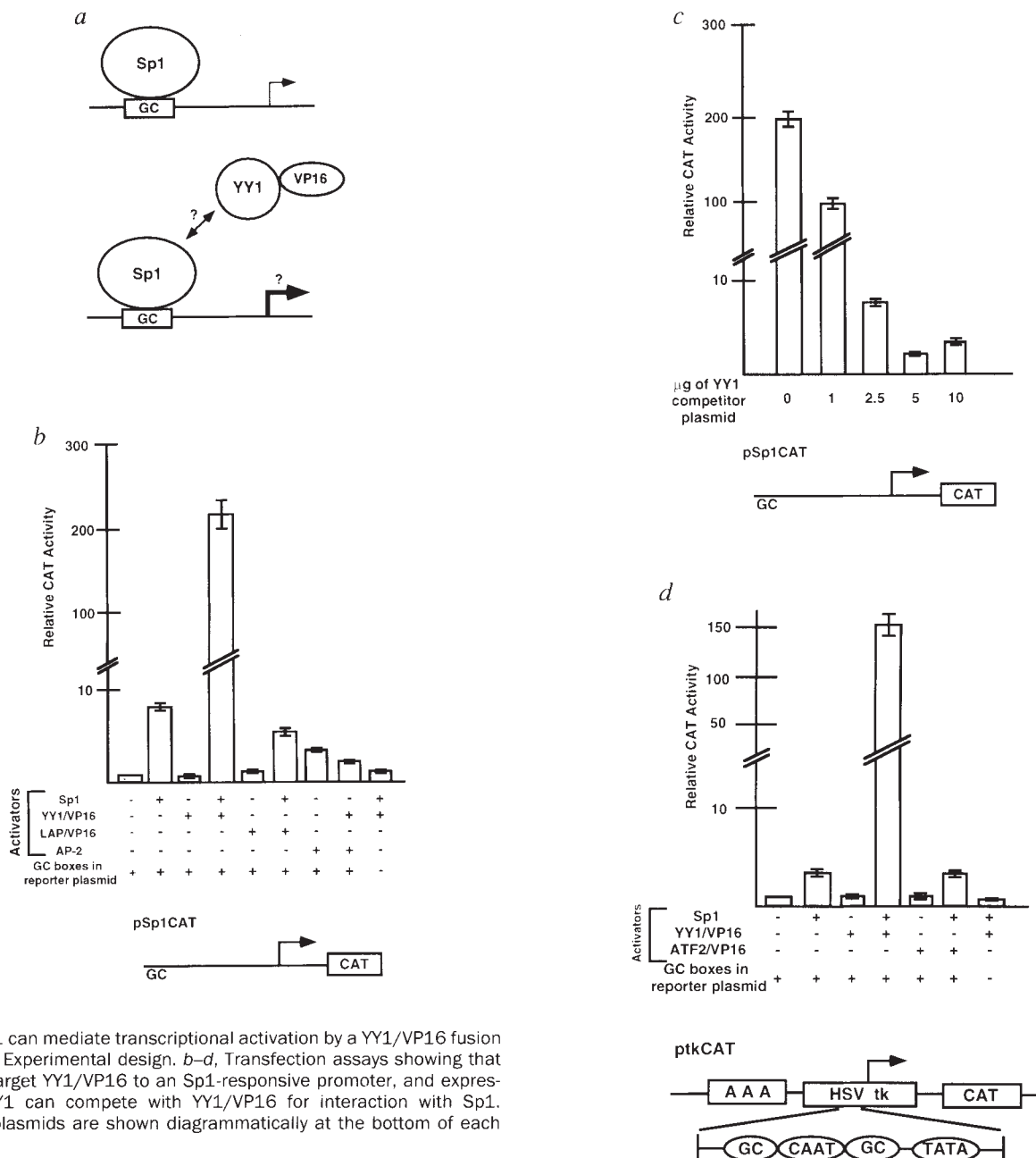


FIG. 2 Sp1 can mediate transcriptional activation by a YY1/VP16 fusion protein. *a*, Experimental design. *b–d*, Transfection assays showing that Sp1 can target YY1/VP16 to an Sp1-responsive promoter, and expression of YY1 can compete with YY1/VP16 for interaction with Sp1. Reporter plasmids are shown diagrammatically at the bottom of each panel.

METHODS. Schneider-2 cells were cotransfected with plasmids directing the synthesis of Sp1 (1 μg DNA), YY1/VP16 (2.5 μg DNA), LAP/VP16 (2.5 μg DNA), ATF2/VP16 (2.5 μg DNA), AP-2 (1 μg DNA) and a CAT reporter (2.5 μg DNA), using the calcium phosphate method¹⁷. CAT assays were done and results presented as described in Fig. 1 but with 30-min reactions. All transfections were normalized to equal amounts of DNA with parental expression vectors. The reporter plasmid pSp1CAT is described in Fig. 1. The reporter plasmid that does not carry GC boxes in *b* was constructed by ligating the CAT reporter gene to pSP72 (Promega). ptkCAT and the reporter that does not carry GC boxes in *d* have been described⁹. Sp1 or YY1 was expressed from pAct-Sp1 or pAct-YY1, respectively, which were constructed by inserting the cDNAs downstream of the *Drosophila* actin 5C promoter in pAct5CPPA¹⁸.

YY1/VP16 was expressed from pADH-YY1/VP16, which was constructed by joining the YY1 coding region in-frame with the activating domain of VP16 (amino acids 413–490) and then placing the recombinant segment downstream of the *Drosophila* alcohol dehydrogenase promoter in pADH¹⁹. LAP/VP16 was expressed from pADH-LAP/VP16, which was constructed by placing LAP348 DNA⁷ in pADH. AP-2 was expressed from pADH-AP2¹⁹. ATF2/VP16 was expressed from pADH-ATF2/VP16, which was constructed by placing the DNA encoding ATF2/VP16²⁰ in pADH. This plasmid encoding ATF-2/VP16 produced a functional product because it strongly activated a promoter containing ATF binding sites in Schneider-2 cells (data not shown).

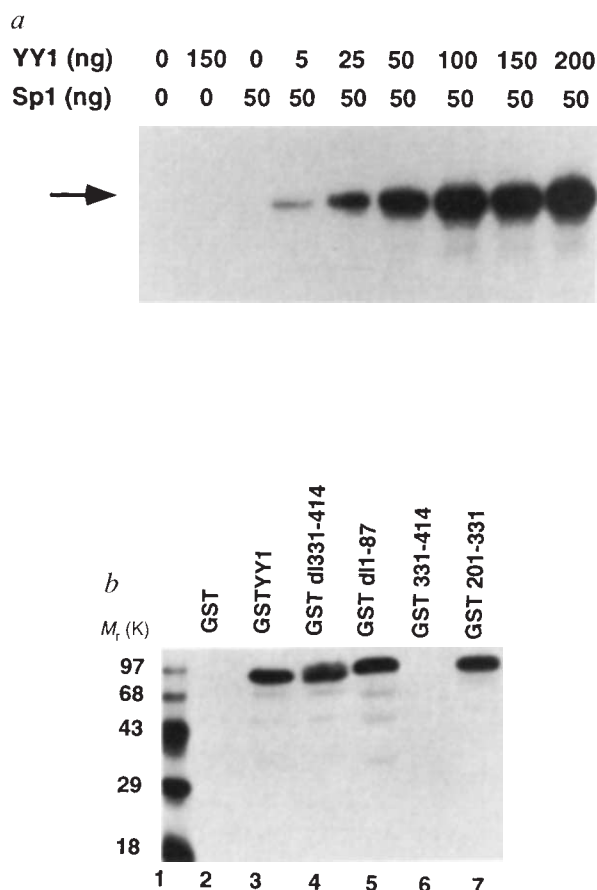


FIG. 3 Sp1 and YY1 can interact physically. **a**, Double-stranded oligodeoxynucleotide containing an Sp1 binding site can be immunoprecipitated by a YY1-specific antibody in the presence of purified YY1 and Sp1. The size of the ^{32}P -labelled oligodeoxynucleotide is indicated by an arrow. The protein amounts used in each reaction are indicated at the top. After incubation of the oligodeoxynucleotide with proteins, the mixture was immunoprecipitated with a YY1-specific polyclonal antibody. DNA coprecipitated in the immune complexes were resolved on a 10% polyacrylamide gel. **b**, Capture of *in vitro* translated Sp1 protein with GST-YY1 fusion proteins. The size of marker polypeptides is indicated.

METHODS. **a**, Synthetic oligodeoxynucleotide 5'-ATTGATCGGGGCGGG-GCGAGC-3' and its complement were labelled with [γ - ^{32}P] using T4 polynucleotide kinase. Purified Sp1 protein (Promega) was incubated with 20 fmol labelled DNA in the presence or absence of various amounts of YY1 (purified from *E. coli* expressing the YY1 cDNA⁵) in binding buffer consisting of 12 mM HEPES, pH 7.9, 10% glycerol, 5 mM MgCl₂, 60 mM KCl, 1 mM dithiothreitol (DTT), 50 $\mu\text{g ml}^{-1}$ bovine serum albumin, and 10 $\mu\text{g ml}^{-1}$ salmon sperm DNA. Reactions were incubated at 37 °C for 30 min. Immunoprecipitation and electrophoresis were essentially as described¹¹, using a purified polyclonal anti-YY1 antibody³ and a buffer containing 250 mM NaCl, 50 mM HEPES, pH 7, 5 mM EDTA, 1 mM PMSF, 0.5 mM DTT, and, 0.1% NP-40. **b**, GST-YY1 contained the YY1 cDNA in pGEX-2T (Pharmacia), and derivatives expressing portions of YY1 fused to GST were prepared using restriction sites to subdivide the YY1 coding region. Sp1 mRNA was transcribed *in vitro*, and translated in a rabbit reticulocyte lysate in the presence of ^{35}S -methionine. Sp1 (20,000 c.p.m.) was added to the indicated GST fusion protein (~1 μg) bound to glutathione-Sepharose beads in incubation buffer (300 μl ; 50 mM KCl, 0.1% Tween 20, 40 mM HEPES, pH 7.5, 5 mM 2-mercaptoethanol, 0.5% nonfat dried milk). Mixtures were incubated at 4 °C for 60 min, the beads were pelleted by centrifugation and washed two to three times in buffer containing 100 mM KCl; bound proteins were removed from the beads by boiling in sample buffer containing 1% SDS, and the eluate was analysed by electrophoresis in a 10% polyacrylamide gel. Although the assays displayed used a washing buffer containing 100 mM KCl, complexes were resistant to washes with 500 mM KCl (data not shown).

action fits well with our observation that Sp1 binding sites can greatly increase the activity of the YY1 initiator sequence (Fig. 1; ref. 3). Physical contact also provides a simple mechanism by which a stimulatory signal can be transduced between Sp1 bound to an upstream sequence and YY1 located in the midst of the basal transcriptional initiation machinery. Other initiator elements that do not appear to bind YY1^{1,3} are also synergistically activated when placed downstream of Sp1 binding sites, implying that Sp1 might interact with proteins other than YY1 that function at initiator elements. □

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Crystal structure of the DsbA protein required for disulphide bond formation *in vivo*

Jennifer L. Martin^{*†}, James C. A. Bardwell^{†‡} & John Kuriyan^{*}

^{*} The Rockefeller University and Howard Hughes Medical Institute, 1230 York Avenue, New York, New York 10021, USA

[†] Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115, USA

PROTEINS that contain disulphide bonds are often slow to fold *in vitro* because the oxidation and correct pairing of the cysteine residues is rate limiting^{1,2}. The folding of such proteins is greatly accelerated in *Escherichia coli* by DsbA^{3,4}, but the mechanism of this rate enhancement is not well understood. Here we report the crystal structure of oxidized DsbA and show that it resembles closely the ubiquitous redox protein thioredoxin⁵, despite very low sequence similarity. An important difference, however, is the presence of another domain which forms a cap over the thioredoxin-like active site of DsbA. The redox-active disulphide bond, which is responsible for the oxidation of substrates, is thus at a domain interface and is surrounded by grooves and exposed hydrophobic side chains. These features suggest that DsbA might act by binding to partially folded polypeptide chains before oxidation of cysteine residues.

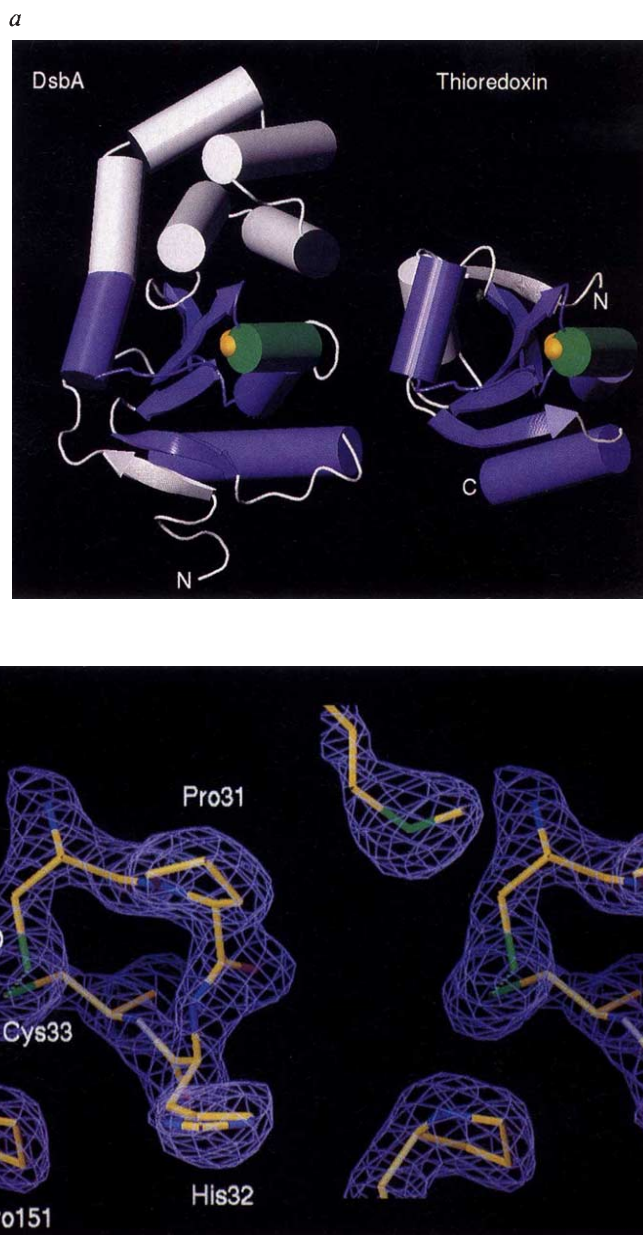
The structure of the 189 residue protein DsbA (Fig. 1) was determined at 2 Å resolution by a combination of multiple iso-

[†] Present addresses: Centre for Drug Design & Development, University of Queensland, Brisbane 4072, Australia (J.L.M.); Institut für Biophysik und Physikalische Biochemie, Universität Regensburg, 8400 Regensburg, Germany (J.C.A.B.).

FIG. 1 a, Comparison of the structures of DsbA and *E. coli* thioredoxin⁸. The figure was generated using program O²⁹. In this orientation of DsbA, domain A is at the bottom, domain B at the top and the active site in the centre. N and C termini are labelled, except for the C terminus of DsbA, which is hidden behind strand $\beta 1$. Parts of the two structures that are similar are purple and those that are different are white. Helix $\alpha 1$ of DsbA, and its equivalent in thioredoxin, is highlighted in green with the sulphur atoms of the disulphide bond shown as yellow spheres. b, Stereo diagram of a difference electron density map at 2.0 Å resolution for active site residues of DsbA. Contour levels at 4σ above the mean are shown. The map was generated for the final refined structure using Fourier coefficients $(|F_o| - |F_c|) \exp(i\alpha_c)$, where F_o and F_c are the observed and calculated structure factors, respectively, and α_c is the calculated phase. Residues Cys 30, Pro 31, His 32, Cys 33, Met 64 and Pro 151 were omitted from the structure factor calculation. Atoms are coloured yellow for carbon, red for oxygen, blue for nitrogen and green for sulphur. A green dashed line indicates the disulphide bond.

METHODS. The quality of the initial MAD/MIR combined phases is reflected in the fact that 85% of the sequence, including side chains, of the two DsbA molecules in the asymmetric unit was readily built²⁹ into the first solvent-flattened map³⁰. The complete atomic model was subsequently built into a map generated using modified phases from SQUASH³¹. This model was refined against native data using X-PLOR³². The final structure includes 195 water molecules and all residues except the C-terminal lysine (residue 189) in both molecules of the asymmetric unit. The density is continuous except for two loop regions (residues 13–18 and 51–55) in both molecules. The *R*-factor is 16.9% for data between 6 Å and 2 Å, including 25,426 structure factors with amplitudes greater than 2σ , which corresponds to 84% of the unique data. The deviations from ideal bond lengths and angles are 0.011 Å and 1.6°, respectively. The two molecules in the asymmetric unit are

morphous replacement (MIR) and multi-wavelength anomalous diffraction (MAD) (Table 1). A notable feature is the presence of a compactly folded helical domain that is inserted into the sequence of another domain, which resembles thioredoxin. The thioredoxin domain (domain A) of DsbA is therefore discontinuous in sequence, and is formed by residues 1 to 62 and 139 to 189 (Fig. 2). It consists of a five-stranded β -sheet ($\beta 1$ – $\beta 5$) bounded on one side by helices ($\alpha 1$, $\alpha 1'$ and $\alpha 7$) that are roughly parallel to the strands, and on the other side by a helix ($\alpha 6$) oriented almost perpendicular to the direction of the strands. The long helix, $\alpha 6$, is considered to span the boundary between domain A and the helical domain (domain B) because its C terminus aligns with a shorter helix in thioredoxin. Domain B is thus formed by residues 63 to 138 and is embedded into the sequence of domain A. It consists of an antiparallel three helix bundle ($\alpha 2$ – $\alpha 4$), with two additional helices ($\alpha 5$ and the N term-



structurally similar with an r.m.s. difference of 0.95 Å for all 188 *Ca* atoms, and 0.4 Å for 167 *Ca* atoms, (excluding residues 1–2, 13–18, 51–55, 165–171 and 188 that are at the termini or in the loops). Coordinates have been deposited with the protein data bank³³.

inus of $\alpha 6$) that wrap around the bundle and form a connection back to domain A. The antiparallel three helix bundle is an unusual fold and other examples of this have been reported only recently, in a synthetic peptide⁶ and a 38-residue pheromone⁷.

The structural similarity between domain A and thioredoxin is striking (Fig. 1a), 71 of the 108 residues of *E. coli* thioredoxin⁸ can be aligned with residues in domain A with an r.m.s. deviation in *Ca* positions of 1.8 Å. Despite low overall sequence identity (10%) between domain A and thioredoxin, there are two separate regions that have local sequence similarity (Fig. 2b), suggesting divergence from a common ancestor. The first region incorporates strand $\beta 2$ and the active site disulphide in helix $\alpha 1$. The second region of similarity is within a loop connecting helix $\alpha 6$ with strand $\beta 4$. This region also forms part of the active site, but is distant in sequence from the first. The sequence of the loop is highly conserved in thioredoxins⁵ and homologues of

FIG. 3 Molecular surface and charge distribution of DsbA, generated using GRASP³⁴. Left, an orientation similar to that in Fig. 1a. Right, a view obtained by rotating by 90° about the vertical axis. The molecular surface is coloured according to charge; white is uncharged, red is negative (Asp and Glu residues) and blue is positive (Arg and Lys residues). Features of the structure of DsbA that might be important for binding or recognition of proteins or polypeptides are indicated. As expected from biochemical data²⁰, only one of the two cysteines (Cys 30) of the disulphide is accessible to solvent. This sulphur atom is indicated by S in the figure at left. A number of exposed hydrophobic residues are located close to the active site. These residues form a contiguous exposed hydrophobic surface, labelled hydrophobic patch (see text). There are two grooves present on the surface of DsbA. Groove 1 is formed in domain A by a lengthening of the loop connecting strand $\beta 5$ and the C-terminal helix ($\alpha 7$) and a shift in the position of this strand, with respect to the thioredoxin structure. Part of this groove is lined with hydrophobic residues (see text). Groove 2, formed at the interface between domains A and B, is on the opposite side of the molecule to the active site. A cluster of acidic residues, Glu 37, Glu 38, Glu 85, Asp 86, Glu 94, Asp 107 and Asp 110, near groove 2 is indicated in the figure as an acidic patch.

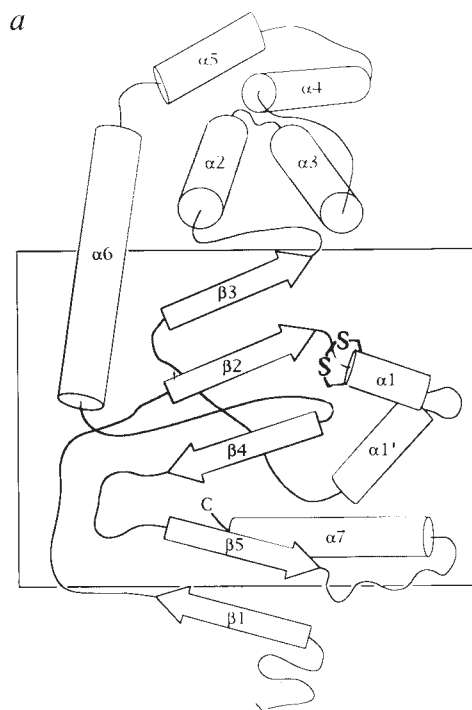
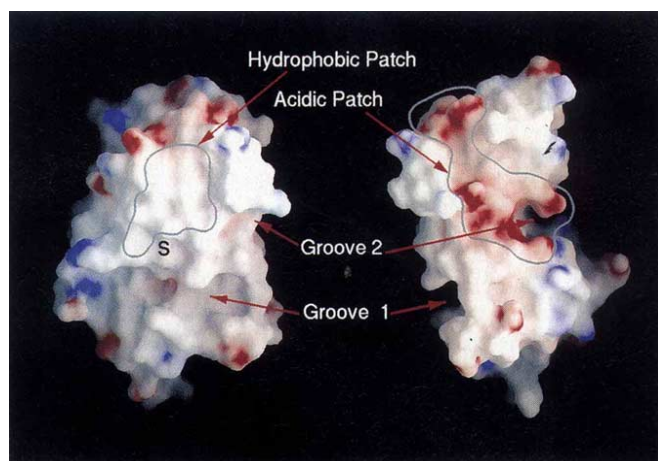
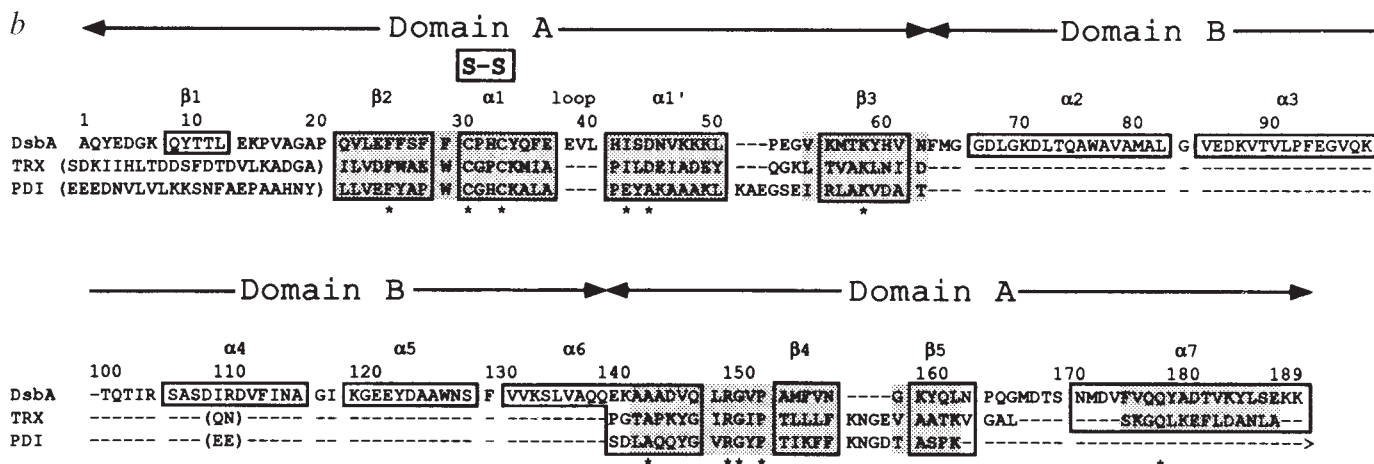


FIG. 2 a, Schematic diagram of the secondary structure connectivity of DsbA, in roughly the same orientation as in Fig. 1a. The boxed part of the diagram indicates elements that are also found in thioredoxin. Domain A includes all of the boxed region as well as the N-terminal strand $\beta 1$. Domain B includes the unboxed elements, at the top of the diagram. The redox-active disulphide bond of DsbA is indicated by S-S in helix $\alpha 1$. b, Sequence alignment of DsbA and *E. coli* thioredoxin (TRX), based on the structural alignment. Also shown is the first thioredoxin domain of rat PDI^{5,25}. The domain organization of DsbA is depicted with arrows and the two cysteines of the disulphide are indicated by the label S-S above the sequence. Shaded regions highlight the 71 residues used to align the structures of DsbA and thioredoxin, and asterisks denote the 11 that are identical. Residues given in parentheses are not aligned with DsbA. Secondary structural elements are labelled and boxed. Helices $\alpha 1$ and $\alpha 1'$ are separated by a three-residue loop in DsbA, whereas in thioredoxin the corresponding elements form one continuous helix that is bent by a proline.



DsbA⁹. In both DsbA and thioredoxin the loop contains a proline that is in the less common *cis* conformation and which packs against the redox-active disulphide bond (Fig. 1b). The first region of sequence similarity has been noted previously³ and was used as part of a sequence alignment, in combination with secondary structure prediction, to suggest that DsbA has a thioredoxin fold¹⁰. Although that conclusion is now shown to be correct, the overall sequence alignment and secondary structure assignment is wrong because the insertion of domain B was not accounted for. Specifically, the second region of sequence similarity is only apparent after alignment of the structures, because the insertion of domain B in DsbA occurs between these two regions.

The thioredoxin fold has been identified in the three-dimensional structures of several proteins: glutaredoxin^{11–13}, glutathione peroxidase¹⁴, glutathione *S*-transferase^{15,16} and now DsbA. The comparison between DsbA and glutathione peroxidase is particularly interesting because both proteins have insertions of similar size (~80 residues) at the same position in the thioredoxin fold. Unlike DsbA, the insertion in glutathione peroxidase does not form a distinct compact structure, but rather wraps around the thioredoxin-like core and forms the inter-subunit contact surface of the tetrameric molecule. Clearly, the thioredoxin fold is a versatile scaffolding that has been used in nature for diverse functions.

Disulphide bonds in folded proteins are usually buried¹⁷, and often in hydrophobic environments. Thus, the ability of DsbA to form disulphide bonds in other proteins suggests that it might interact with hydrophobic regions of polypeptide chains. In addition, reduced DsbA may associate with an accessory protein to re-oxidize the active site disulphide^{18,19}. Although it is not known whether DsbA has any peptide-binding activity, several features of the three-dimensional structure are suggestive of peptide- or protein-binding surfaces (Fig. 3). In domain A, a long and relatively deep groove (groove 1) runs below the active site and has no equivalent in thioredoxin. Importantly, a section of groove 1 close to the disulphide is lined by a cluster of hydrophobic side chains (Phe 36, Leu 40, Pro 151, Pro 163, Met 171, Phe 174 and Val 175). In addition, a number of solvent-accessible residues from both domains (Phe 29, Phe 63, Met 64, Gly 65, Gly 66, Leu 68, Val 96, Ala 105 and Val 150) form a hydrophobic patch on the other side of the disulphide bond. The positioning of domain B with respect to domain A therefore places the active-site disulphide in the centre of a relatively extensive hydrophobic protein surface, which could provide stabilizing interactions for partially folded substrates. Domain B also forms a second prominent groove (groove 2) at the domain interface, which is bordered at one edge by a cluster of acidic residues.

Disulphide bonds in proteins are generally stabilizing and non-reactive. But oxidized DsbA is less stable than the reduced form, and its disulphide bond is very reactive^{20,21}. DsbA is thus a strong oxidizing agent, consistent with its ability to form disulphide bonds in other proteins. It has been suggested that the higher redox potential of DsbA²² relative to *E. coli* thioredoxin²³, which corresponds to a destabilization of the oxidized form in DsbA by $-6.9 \text{ kcal mol}^{-1}$ (refs 20, 21), is due to a strained conformation of oxidized DsbA²¹. This strain is unlikely to be due to local deformations, because the 14 atoms that form the disulphide ring in the two proteins have virtually identical conformations (r.m.s. deviation 0.1 Å). Comparison of the structures of oxidized and reduced DsbA may help explain the atypical behaviour of the disulphide, but suitable crystals of reduced DsbA are not yet available²⁴.

Protein disulphide isomerase (PDI) helps the formation of disulphide bonds in eukaryotic cells. Although the structure of this large multifunctional enzyme is unknown, sequence alignment predicts that each molecule of PDI contains two thioredoxin-like domains²⁵ (Fig. 2b). Thus, the bacterial and eukaryotic proteins that form disulphide bonds both use thioredoxin modules. Thioredoxin by itself may be too small to inter-

TABLE 1 Crystallographic data

Wave-length (Å)	Observed ratio of anomalous diffraction differences				Scattering factors	
	$\lambda 1$	$\lambda 2$	$\lambda 3$	$\lambda 4$	f'	f''
	0.9873 Å (0.038)	0.9795 Å	0.9791 Å	0.9642 Å		
$\lambda 1$	0.045	0.056	0.054	0.044	-3.7400	1.0605
$\lambda 2$		0.060 (0.042)	0.051	0.066	-9.5006	2.9763
$\lambda 3$			0.085 (0.048)	0.063	-7.5235	5.6937
$\lambda 4$				0.069 (0.043)	-3.5600	3.7100
Phasing statistics						
	Figure of merit			Phase difference		
MIR	0.54			63°		
MAD	0.64			57°		
MIR/MAD	0.72			52°		

Bijvoet differences from MAD data measured from selenomethionyl (Se-Met) DsbA are given in the diagonal elements of the table, with centric values (a measure of the noise in the anomalous signals) in brackets³⁵. Dispersive differences are given as off-diagonal elements. The refined values of the scattering factors are listed for each wavelength. Values of f' and f'' at $\lambda 4$ were fixed at their calculated values for this refinement. The figures of merit for reflections between 15 and 2.5 Å for MIR, MAD and combined phases are listed. R.m.s. phase differences between solvent-flattened phases and phases calculated from the refined atomic model are also given. Methods: Purification and crystallization of DsbA and Se-Met DsbA has been described previously²⁴. Crystals belong to space group C2, with unit cell parameters $a = 117.7 \text{ Å}$, $b = 65.1 \text{ Å}$, $c = 76.4 \text{ Å}$ and $\beta = 126.3^\circ$, and two molecules in the asymmetric unit. An osmium derivative was formed by soaking a native crystal in a solution of 5.6 mM K_2OsCl_6 for 53 h. MIR X-ray intensity measurements were made on an R-Axis IIC area detector connected to a Rigaku RU200 rotating anode X-ray source. One crystal was used for each data set. Native data are 87% complete to 2 Å, with 1.7-fold redundancy and an overall $R_{\text{sym}}(I)$ of 3.5%. For the resolution shell 2.0 to 2.25 Å, native data are 70% complete with $I/\sigma(I)$ of 6.6. Data for Se-Met and osmium derivatives are 94% complete to 2.5 Å, with fourfold redundancy and $R_{\text{sym}}(I)$ of 6.3% and 5.6%, respectively. For the resolution shell 2.5–2.75 Å, $I/\sigma(I)$ and completeness are 6.7 and 85% for Se-Met, and 5.25 and 84% for osmium data. Anomalous difference measurements were made for both derivatives. All data were integrated, scaled and reduced using the PROCESS package (Rigaku, Japan). The positions of two osmium sites were determined using MULTAN²⁶. An additional 2 osmium sites and 12 selenium positions were found by difference Fourier methods. Refinement of heavy atom parameters²⁷ resulted in phasing powers to 2.5 Å of 1.03 and 1.55 for the Se-Met and Os derivatives, respectively. MAD data were measured from four Se-Met DsbA crystals at the HHMI beamline X4A at the National Synchrotron Light Source (Brookhaven, NY) using Fuji imaging phosphor plates. The wavelengths used correspond to the inflection point ($\lambda 2$, 0.97946 Å), the peak value ($\lambda 3$, 0.97915 Å) and two remote values ($\lambda 1$, 0.98726 Å) and $\lambda 4$, 0.96423 Å) of the X-ray absorption spectra of a DsbA crystal. Oscillation ranges of 3.5–4°, with 0.3° overlap and a crystal to film distance of 230 mm were used. The images were processed using DENZO (Z. Otwinowski, unpublished) and integrated intensities were scaled and processed as described previously²⁸. $R_{\text{sym}}(I)$ was 6.2% ($\lambda 2$), 7.7% ($\lambda 3$), 6.6% ($\lambda 4$) and 3.8% ($\lambda 1$). 75% of all Bijvoet pairs to 2.5 Å were measured for wavelengths $\lambda 2$, $\lambda 3$ and $\lambda 4$ and 25% for $\lambda 1$. Redundant reflections were merged after scaling and phasing. MAD phases were then combined with MIR phases for electron density map calculations.

act effectively with extended polypeptide chains, as may be required during protein folding and disulphide shuffling. In the case of DsbA, a conspicuous feature is the acquisition of a new domain. As a consequence, the structure of DsbA resembles that of most enzymes, with the active site at the interface between domains. This provides for a more complex and extensive binding surface than seen in thioredoxin, and may allow the accommodation of a wide range of substrates through domain movements. The actual mechanism of the catalysis of disulphide bond formation by DsbA is still unclear, but the three-dimensional structure now makes possible the rigorous testing of the role of specific elements. □

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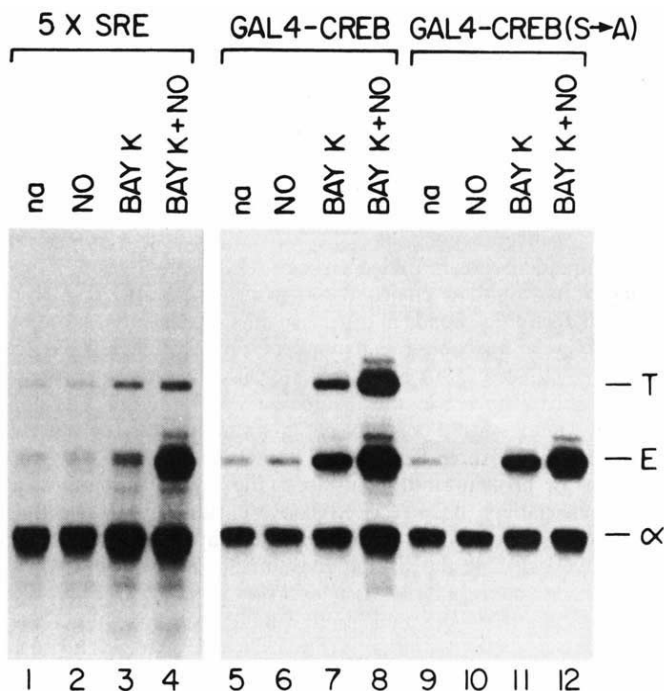
ACKNOWLEDGEMENTS. We thank W. A. Hendrickson and C. Ogata for time on synchrotron beamline X4A which is funded by the Howard Hughes Medical Institute at the Brookhaven National Laboratory; D. Kominos and B. Guenther for help in MAD data collection; W.A.H., W. I. Weis and M. Newman who helped with MAD data processing; R. Glockshuber for communicating unpublished work; Z. Otwinowski for the DENZO program; and T. E. Creighton, X.-P. Kong, E. Rattigan, J. L. Kim, K. L. Clark and S. K. Burley. J.B. was funded by a Helen Hay Whitney Fellowship. Supported in part by grants from the NIH to J. Beckwith and J.K. We thank J. Beckwith and P. Model for helping to start this project.

Amplification of calcium-induced gene transcription by nitric oxide in neuronal cells

Natalia Peunova & Grigori Enikolopov

Nature **364**, 450–453 (1993)

IN this letter an earlier version of Fig. 3c was printed instead of the revised and expanded figure which is discussed in the text and described in the legend. The correct figure is shown here. □



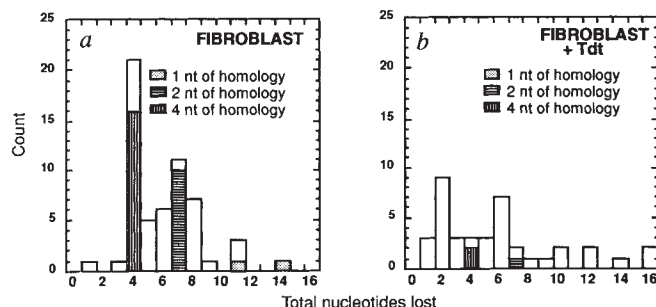
ERRATA

Extent to which homology can constrain coding exon junctional diversity in V(D)J recombination

Rachel M. Gerstein & Michael R. Lieber

Nature **363**, 625–627 (1993)

DURING the production process, the key to Fig. 3 of this letter was inadvertently altered. The correct figure is shown. □



CORRECTION

A new component of the transcription factor DRTF1/E2F

Rowena Girling, Janet F. Partridge, Lasantha R. Bandara, Neil Burden, Nicholas F. Totty, J. Justin Hsuan & Nicholas B. La Thangue

Nature **362**, 83–87 (1993)

THIS letter contains an error in the DP-1 cDNA sequence shown in Fig. 1b which affects the codon reading frame from amino acid 388. The corrected sequence appears below, starting at position 1,217:

ACC OCT GTG TCC TAC GTT GGG GAG GAT GAT GAC GAC GAT GAT TTT AAT GAG AAC GAC GAG GAG GAT
T P V S Y V G E D D D D D D D F N E N D E E D

This correction does not alter any of the conclusions of the paper, although the open reading frame is now 410 amino acids. The corrected sequence has been submitted to the EMBL data base, accession number X72310. □

Parallel genome analysis by two-dimensional DNA typing

E. Mullaart, G. J. de Vos, G. J. te Meerman, A. G. Uitterlinden and J. Vijg

By two-dimensional (2-D) DNA typing a restriction enzyme digest of genomic DNA can be resolved on the basis of both size and base-pair sequence and subsequently analysed by repeat probe hybridization to reveal sequence variants at multiple genomic sites in parallel. The system has been partly automated and allows for large-scale comparative analysis of complex genomes in a cost-effective manner.

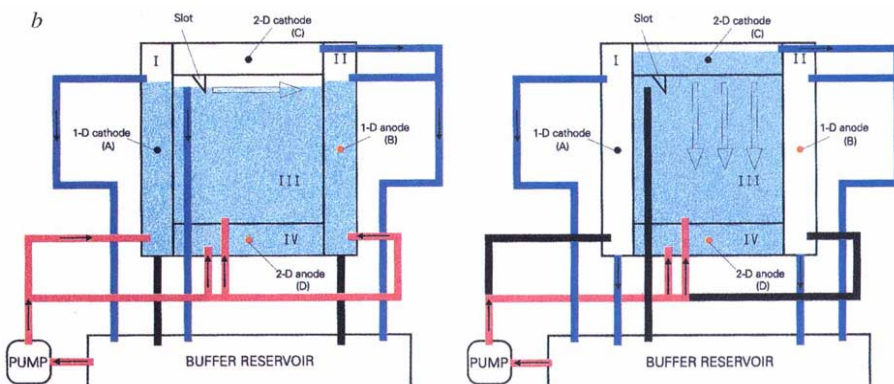
ELECTROPHORETIC separation in two dimensions has found widespread application in protein analysis. Originally the method proved useful in detecting variation in patterns of individual proteins, for example during differentiation¹. The method has since undergone extensive sophistication. Most importantly, the generation of protein databases now makes it possible to screen for intensity differences at spots for which the corresponding proteins have been identified. This as well as a whole range of technical improvements has greatly increased the accuracy of the method since the introduction of the concept independently by O'Farrell² and Scheele³.

The first successful attempt to separate DNA fragments in two dimensions according to independent separation criteria was made by Fischer and Lerman⁴. These authors succeeded in differentiating between an *Escherichia coli* genome with or without lambda phage and laid the groundwork for the successful use of denaturing gradient gel electrophoresis as a mutation detection assay. We have adopted the two-dimensional (2-D) separation system of Fischer and Lerman to separate human genomic DNA restriction enzyme digests followed by hybridization analysis using minisatellite core probes⁵. The large number of data on polymorphic loci obtained in this way could find major applications in genetic mapping and association studies, studies on genetic instability in cancer, analysis of somatic and germinal mutations and many other areas of DNA analysis. Thus far, widespread application of the method has been constrained by the relatively high complexity of the experimental procedure, including the electrophoresis process *per se* and the evaluation of the complicated spot patterns. In an effort to facilitate high-throughput 2-D DNA typing and to provide easy access to the method we have designed a semiautomated system for running multiple 2-D gels without manual interference and an interactive image analysis system to evaluate spot patterns on the basis of internal markers and probe-specific databases of polymorphic loci.

Semiautomated 2-D DNA typing

According to the present protocol for 2-D DNA typing the first and second dimensions are run separately^{4,5}. After separation of the

FIG. 1 Semiautomated two-dimensional (2-D) DNA typing instrument for ten gels. *a*, The electrophoresis unit with the two cartridges. Buffer reservoir, power supply etcetera are not yet built in with this prototype version. *b*, A schematic depiction of the principle underlying the 2-D DNA typing instrument. Electrode switching and buffer level adjustment between the first and second dimension occurs automatically. The tube colours indicate flow directions: red, ingoing; blue, outgoing; and black, closed.



DNA fragments in the first dimension on basis of their length, the lane containing the separation pattern is cut out and placed on a second gel containing a gradient of denaturants (that is, formamide and urea. In this gel (the second dimension) the fragments are separated on basis of their base-pair composition. This procedure is laborious and requires trained personnel. In addition, due to the manual handling of the first dimension lanes, artefacts (stretching and breakage) are often introduced, resulting in a less reproducible separation pattern. The system we designed is an instrument to semi-automatically produce ten 2-D DNA typing patterns at a time with a minimum of manual interference. The prototype system (Fig. 1*a*), which was constructed by Instrumek B.V., Schiedam, The Netherlands, consists of two pairs of electrodes and four separate buffer chambers (schematically depicted in Fig. 1*b*). The gels are cast outside the instrument in a simple gel-casting device using small spacers (1 × 2 × 2 mm) attached in the corners of the glass plates. Once cast the gel-sandwiches are slid between the sili-

cone sealings in the cartridges. The cartridges are then placed in the system without any clamps or screws. Samples are loaded in the V-shaped slots (upper left in Fig. 1*b*) and run first from left to right, horizontally, occupying only the upper part of the gel. At this stage buffer chambers I and II are filled and electrodes A and B are activated. The buffer level in chamber III is just below the top of the gel (see left part of Fig. 1*b*). When this level is too high the separation patterns are deformed due to electric field deviations. A completely empty chamber III, however, would not allow to keep the (elevated) temperature constant.

After the first-dimension run, buffer chambers I and II are automatically evacuated and chamber III is filled (also automatically) to a level just above the top of the gel (see the right part of Fig. 1*b*). Then, by switching to the other pair of electrodes (C and D), the 2nd dimension is run from top to bottom, now occupying the entire gel. To prevent exhaustion, the buffer in these compartments is circulated. The buffer is heated to the desired temperature by a thermostated

heating element in the buffer reservoir. The reservoir is indicated in the schematic depiction (Fig. 1b), but absent in the prototype (Fig. 1a); in the prototype a separate buffer reservoir was used.

The result of a separation of marker DNA fragments (restriction enzyme-digested phage lambda DNA) is shown in Fig. 2. On top the result of a first dimension separation only is shown: a pattern of well-separated vertical sharp bands. After separation of the marker mixture in two dimensions, a pattern of well-separated spots was obtained (Fig. 2).

Less complex separation patterns, like polymerase chain reaction products (PCR) from defined genomic areas or restriction enzyme digests from lower organisms can be directly visualized by staining. Patterns can then be calibrated on the basis of marker fragments and automatically interpreted by comparison with databases of known sequence variants as described below. In 2-D DNA typing of higher organisms marker DNA fragments are mixed with restriction enzyme digested total genomic DNA. After electrophoresis the 2-D separation patterns are transferred to a hybridization membrane by means of electroblotting, allowing multiple sequential hybridizations with different micro- and minisatellite core probes (for a detailed description of the entire procedure, see ref. 6). For each probe, this results in the visualization of hundreds of DNA fragments as spots. By using several different core probes it is possible to screen thousands of polymorphic loci per individual.

Evaluation of spot patterns

Most core-probe-specific 2-D spot patterns are complex and their evaluation is therefore time-consuming and cumbersome. This ob-

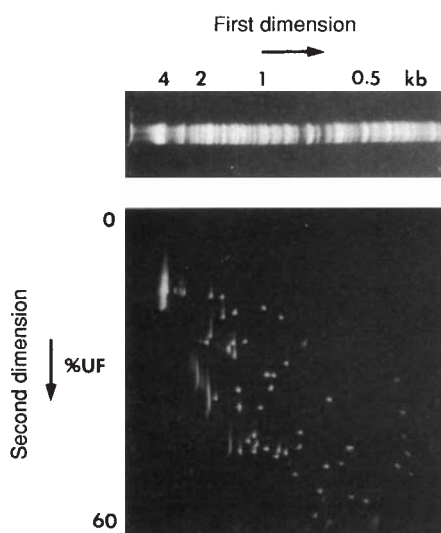
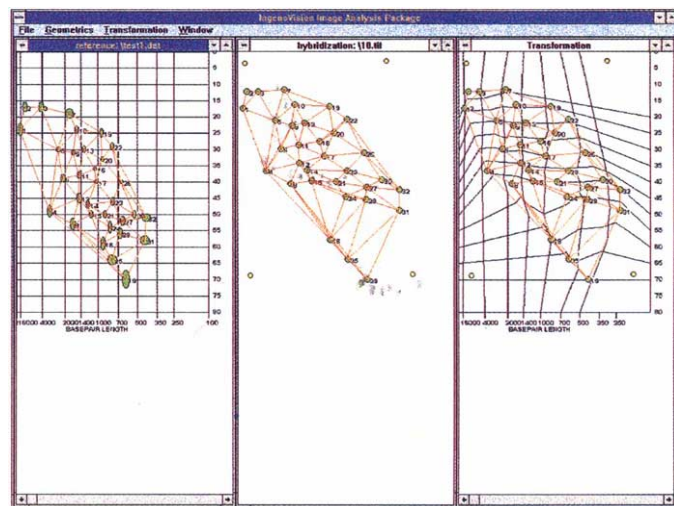


FIG. 2 Ethidium bromide-stained gel pattern after electrophoresis in one (top) and two dimensions of DNA marker fragments using the semi-automatic instrument. The first dimension was run at 200 V (0.35 A) for 3 h at 50 °C; the second dimension was run at 200 V (0.5 A) for 8 h at 60 °C.

FIG. 3 Automatic interpretation of gel patterns on the basis of marker spots. On the left the marker database is shown with the coordinate system. The marker pattern of the sample is shown in the middle. The right panel shows the transformed database.



viates the use of image analysis systems, a great many of which are presently available. We designed a system based on the use of the internal lambda marker fragments shown in Fig. 2. By mixing the markers with each DNA digest to be analysed the resulting spot patterns can be calibrated on the basis of the markers as internal standards. Central to this concept is the generation and use of databases containing x,y-coordinates of selected spots from hybridization patterns of lambda probe and different mini- and microsatellite core probes.

For minisatellite core probe 33.6, for example, a database was generated consisting of 160 spots. These spots were selected as representing highly informative marker loci on the basis of their segregation behaviour in CEPH (Centre d'Etude du Polymorphisme Humain) pedigrees. By analysing co-segregation with known markers in two CEPH pedigrees, specific chromosomal positions could be assigned to several of these spots (E. Mullaart *et al.*, in preparation).

The 33.6 database pattern can be transformed to any new incoming 33.6 hybridization pattern on the basis of the marker spots. These marker spots are detected in the sample pattern by a final round of hybridization with lambda probe. Transformation is performed by triangular image matching, using a computer program running under Windows (G. J. te Meerman, in preparation). First, the marker spots in the sample pattern (Fig. 3, middle) corresponding to those in the marker database (Fig. 3, left) are indicated on the screen. The database marker pattern is then transformed together with the coordinate system to match the sample marker pattern (Fig. 3, right). With these new sample-specific coordinates the core-probe-specific databases are transformed to match accurately the core-probe hybridization patterns of the sample. A manual or automatic search of all possible scoring positions rapidly provides information about the presence or absence of particular spots in the sample hybridization patterns.

The above system can be used, for example, to localize rapidly all possible genomic changes in particular tumours^{7,8}. Another application is in linkage or association studies to identify genetic traits. For example, data obtained from pedigree analyses can be directly entered into linkage programs. It can easily be calculated that ten re-hybridizations with probes each detecting 160 informative spots allows one to scan the genome at 1,600 loci. In this respect it should be noted that as long as spot interrelationships like allelism and haplotypes have not been established 2-D typing is essentially a dominant marker system and therefore less informative⁹. Further co-segregation studies with the CEPH panel should reveal such relations thereby increasing the amount of information that can be obtained from the system.

Discussion

The size and complexity of higher animal genomes requires repetitive testing of multiple sites to assess individual variation. A typical example is CA-repeat typing using PCR, which has almost become a method of choice in linkage analysis and loss of heterozygosity typing of tumours. We found that a parallel approach rather than a serial one is at least theoretically attractive. The concept of high-resolution 2-D DNA typing based on independent electrophoretic separation in combination with the minisatellite core probe principle as first described by Jeffreys¹⁰ was initially found to be complicated and difficult to implement in many laboratories. Our goal was to remove as much as possible the technical hindrances blocking large-scale application of the method. The first steps in automation that we have introduced should allow easy implementation and increased throughput of the method. The database concept for automatic evaluation of presence or absence of particular spots can be linked to other databases containing genome-specific sequence and physical map information. Such information is presently accumulating, especially for the human genome, which

will greatly contribute to the future applicability of a system that allows the rapid assessment of large numbers of genetic loci.

Finally, both the automated 2-D electrophoresis unit and the image evaluation software were specifically designed for 2-D DNA typing, that is, scanning of the higher animal genome for polymorphic variation. However, the system should find applications also in physical mapping, medical diagnostics and sequencing by hybridization. □

E. Mullaart, G. J. de Vos, A. G. Uitterlinden and J. Vijg are at Ingenu B. V., PO Box 685, 2300 AR Leiden, The Netherlands and G. J. te Meerman is in the Department of Medical Genetics, University of Groningen, A. Deusinglaan 4, 9713 AW Groningen, The Netherlands. For more information, fill in reader service number 100.

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DNA snippets

Featured this week — avidin resin, vector systems, kits galore, assorted gadgets and gismos for yeast geneticists, and an electroporation system.

TROPIX has developed a new chemiluminescent 1,2-dioxetane **enzyme substrate for β -D-glucuronidase** (Reader Service No. 101). The new substrate, Glucuron, enables researchers to detect this enzyme in a non-isotopic assay system. It has been incorporated into the company's GUS-Light kit for the assay of β -D-glucuronidase in such applications as the quantitative detection of the GUS gene as a reporter of gene expression. The GUS gene fusion system has become an increasingly important tool for reporting activities of other genes of interest, particularly in plant cells, where effectively no endogenous β -D-glucuronidase exists.

Promega's new SoftLink soft-release **avidin resin** is said to provide a quick, resilient, convenient and non-denaturing means of purifying biotinylated molecules (Reader Service No. 102). The resin is a rigid acrylic polymer functionalized with monomeric avidin. Unlike tetrameric avidin, which Promega says does not allow release of biotinylated molecules, except under strongly denaturing conditions, the monomeric avidin on the SoftLink resin allows reversible binding under mild elution conditions (such as 5 mM biotin). As a consequence, isolation and purification systems can be designed that are highly specific, yet mild, for sensitive biological materials. The resin is stated to bind reversibly 25-40 nmol of biotinylated protein per millilitre of SoftLink resin. Promega says that it can be regenerated ten times without loss of binding capacity and is resistant to a wide range of conditions. The resin can be used in column or batch purification; applications include purification of bio-tinylated fusion proteins and biotinylated antibodies.



SoftLink binding agent.

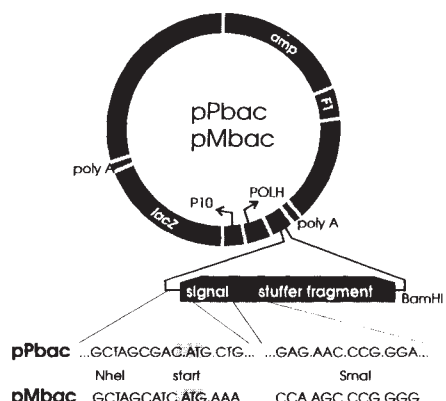
Oncor has increased its range of **human cosmid probes**, obtainable through Alpha Laboratories, which can be used for cytogenetic research, specific translocations, assessing chromosomal deletions and structural rearrangements, and assigning genes to particular chromosomal regions (Reader Service No. 103). The selection includes: Prader-Willi and Angelman syndromes; Charcot-Marie-Tooth (CMT1/Type A); chromosome 13 specific cosmid; chromosome 21/Down syndrome critical region

cosmid; Miller-Dieker cosmid/chromosome 17 α satellite; cri du chat; Wolf-Hirschhorn cosmid/chromosome 4 α satellite and Di George syndrome. Two newly released probes for cancer research are the Her 2/neu cosmid and N-Myc cosmid for *in situ* hybridization.

Amicon's new **centrifugal concentrator**, the Centriprep-50, provides a 50,000 molecular weight cut-off for concentrating, purifying or desalting samples of up to 15 ml (Reader Service No. 104). The non-fouling design makes it suitable for concentrating and desalting particle-laden samples; it is intended for the recovery of biomolecules from cell culture supernatants, lysates, extracts or other biological samples. Amicon says that samples of up to 15 ml can be rapidly concentrated to as little as 0.65 ml in 20-45 min. Integral deadstop protection is designed to prevent filtration of the sample to dryness. The unit's low-adsorptive membrane provides over 90 per cent protein recovery and complete desalting with minimal denaturation or shear, the company says. Disposable Centriprep concentrators are supplied ready-to-use with a cap to prevent aerosol contamination and sample spillage during centrifugation. They can be used in most standard centrifuges with fixed-angle or swinging-bucket rotors and a 50-ml tube adapter.

Vector systems

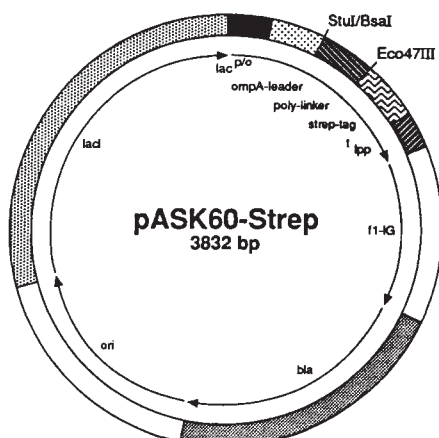
Stratagene has developed two new **baculovirus transfer vectors** that are designed to optimize the production of recombinant proteins in insect cells by directing secretion of the expressed protein into the cell supernatant (Reader Service No. 105). The pPbac and pMbac transfer vectors were developed by inserting the human placental



Vector map of Stratagene's new baculovirus transfer vectors.

alkaline phosphatase or melittin signal sequence, respectively, into the pJVP10Z transfer vector, downstream from the potent polyhedrin promoter. Stratagene says that several proteins have been successfully expressed and secreted using these signal sequences. By forcing secretion into the supernatant, the baculovirus transfer vectors promote efficient production of normally nonsecretory proteins, of fragments or domains of proteins. The vectors also facilitate downstream processing (that is, purification) of the desired protein, which is further simplified by using a serum-free culture medium. In addition to enhancing protein function and stability and increasing yields, insect cell expression systems eliminate the solubility problems associated with prokaryotic expression. Insect cell lines contain the post-translational modification machinery responsible for correct folding, disulphide formation and glycosylation, assuring the production of biologically active protein.

Biometra says that its new Strep-tag kit can be used for the **expression of foreign genes in *Escherichia coli*** and their purification under mild conditions in a one-step



Biometra's cloning and expression vector pASK60-Strep consists of an origin of replication from the pUC family of plasmids (ori), an ampicillin resistance gene (bla), an f1 origin of replication for the preparation of single-stranded plasmid DNA (f1-IG), the Lac repressor gene (lacI), and an expression cassette under control of the lac promoter/operator.

procedure (Reader Service No. 106). The system is based on the expression vector pASK60-Strep and it enables the IPTG-inducible expression of foreign genes in *E. coli* and their secretion by means of the OmpA signal peptide. The Strep-tag sequence is encoded at the 3' end of the polylinker. Induction of the expression is followed by protein secretion into the periplasm where the signal peptide is cut off. The recombinant protein is purified directly by affinity chromatography from the periplasmic cell extract. The protein is bound

quantitatively to immobilized streptavidin at the Strep-tag. The system is recommended for all applications where mild purification of the protein of choice is important.

■ In situ hybridization

Novagen's SureSite II System contains **pre-tested reagents for in situ hybridization**, newly configured into two modules (Reader Service No. 107). The new kit is similar in application to the original SureSite Kit in that it is designed for the use of ³⁵S-labelled antisense probes to localize gene expression in tissues. The system is comprised of a transcription module and a hybridization kit. The T7 Probe Kit contains sufficient reagents for the synthesis of 50 probes. It is designed for use with DNA templates having a T7 promoter positioned to produce antisense RNA during transcription with T7 RNA polymerase. The Hybridization Reagents Kit contains sufficient reagents for prehybridization, hybridization and post-hybridization washing of up to 50 standard microscope slides. To permit maximum flexibility, each kit module and all the individual components are available separately.

Seescan has developed a new **package to enable automated differential counting** of immunolabelled, counterstained cells in sections or cytopins (Reader Service No. 108). The routines enable a total cell count, the subset of cells that is positively labelled and the area over which the count is taken to be quickly determined. This forms part of image analysis routines for use in immunohistochemistry and immunocytochemistry and *in situ* hybridization. The system is designed to work with samples prepared using a blue nuclear counterstain and either horseradish peroxidase or alkaline phosphatase as the immunological stain, although it can be adapted to suit user requirements. Cells are counted from morphological features revealed by the counterstain using statistical pattern recognition techniques. Once detected, these cells are checked to ascertain whether they are above or below an immunostaining threshold predefined by the user. As only the counterstain information is used to identify cells, an unbiased and repeatable count is said to be obtained. Tests made using similar routines for automatic differential cell counting of immunolabelled macrophages and lymphocytes in bronchial lavage samples have shown a high degree of repeatability, says Seescan.

■ Assorted kits

The new Micromix kit from Talent is designed to provide a rapid procedure for the **isolation of genomic DNA** from 200 µl of whole blood or up to five million cultured cells or tissue (Reader Service No. 109). Said to take 10 min, the simple process can be carried out in a 2 ml Eppendorf tube and a centrifuge, and does not require the use of phenol. The method is based on the direct lysis of the sample at 60 °C in the presence of

a cationic detergent mixture. Genomic DNA of a high molecular weight binds to the purification resin and, in the washing step that follows, proteins and RNA are eliminated through a spin filter. The yield of pure DNA, which can be later used for all molecular biology applications (including DNA amplification or restriction endonuclease digestion) is stated to be 1–5 µg. The kit can be used to process fresh anticoagulated whole blood, frozen blood and blood that has been stored at 4 °C for at least 6 months. Sufficient reagents are included in the kit for 100 extractions. The Plasmix kit for the isolation of plasmid DNA works along similar lines. This kit can be used to process quantities from 1.2 ml broth (miniprep), to 15 ml (midiprep), up to 500 ml (maxiprep), with a stated processing time of 20 min.

Du Pont NEN Research Products now offers Reflection **autoradiography film** and Reflection **intensifying screen** designed especially for studies involving the detection of ³²P and chemiluminescence signals



Du Pont's new autoradiography system.

(Reader Service No. 110). The matched film and screen system provides researchers with fast film detection of Southern, northern, colony/plaque and western blots. The fine grain and low fog of Reflection film is said to result in high sensitivity, good resolution and high contrast, making it suitable for single-copy gene detection. Reusable screens are super high-speed products using a new generation rare-earth phosphor. The film can be processed manually or in an automatic processor.

Cambio says that by using its **nick translation kit**, a few nicks per plasmid are sufficient to label virtually all of the plasmid (Reader Service No. 111). Boiling at the end of the reaction will denature the labelled DNA and cause the two DNA strands to separate to give single-stranded DNA that is suitable for a large range of hybridization techniques. The kit contains sufficient rea-

gents for 50 tests and costs UK£85, or £1.70 per test. The kit allows labelling with the user supplying any one of four labelled dNTPs and will work with ^{32}P -, ^{35}S -, ^{33}P -, ^{125}I -, ^3H - or ^{14}C -labelled nucleotides. Cambio says that typically 50–60 per cent of the available labelled dNTP is incorporated into the DNA, providing probes of up to 10^8 c.p.m. μg^{-1} .

The Boehringer Mannheim **chemiluminescence western blotting system** is designed to provide nonradioactive protein detection at the picogram level (*Reader Service No. 112*). Less than 10 pg of antigen can be detected with an exposure of 1 min. Two kits are available. Both are based on the peroxidase substrate luminol — a cyclic diacylhydrazide that forms a radical and then emits light at sites where H_2O_2 is cleaved by peroxidase; the energy transfer is mediated by the enhancer 4-iodophenol. The signals are then captured and recorded on standard X-ray film. The kits differ in the type of antibody supplied: the mouse kit contains anti-mouse IgG peroxidase for use with primary antibodies of mouse origin, and the rabbit kit for use when the antibody originates from rabbit. If other species-specific antibody conjugates are used, the enhanced chemiluminescent substrate, starting solution and blocking reagent can be purchased as a set without the detection antibody.

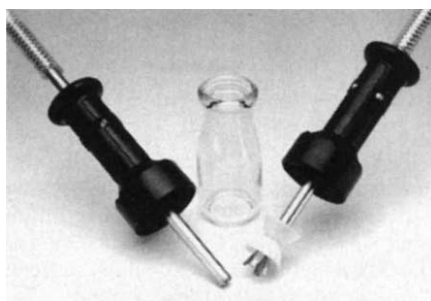
The polymerase formulations in Epicentre's AmpliScribe SP6, T7 and T3 **transcription kits** make them suitable for the high-yield synthesis of RNAs ranging in size from many kilobases to as small as 25 nucleotides (*Reader Service No. 113*). The ability to synthesize small RNAs should be of particular interest to researchers studying ribozyme structure and function. The T7 kit enables the synthesis of up to 150 μg of active ribozyme (50mer) in a 2-h reaction containing 0.25 μg of linearized plasmid as a template. Epicentre says that oligonucleotides carrying both the ribozyme gene and T7 promoter sequences also give good yields of active ribozyme when used as templates. The AmpliScribe system eliminates the need to use hazardous chemicals and the product can be ready in an afternoon.

Amersham says that high-efficiency mutagenesis is possible in a day with its one-tube protocol Sculptor **in vitro mutagenesis kit** (*Reader Service No. 114*). An updated version of Amersham's oligonucleotide-directed *in vitro* mutagenesis kit, Sculptor is said to offer three added advantages: the use of T7 polymerase in the initial extension reaction, enzyme digestion by T5 exonuclease to remove remaining single-stranded template DNA from the double-stranded product of the extension reaction, and a streamlined protocol with the number of additions reduced to a minimum. In addition, Amersham has extended its range of

products for *in situ* hybridization with the launch of two new ^{35}S nucleotides. [^{35}S]-UTP αS can be used to label the RNA probes for this application and has been developed to give both high specific activity and high concentrations to give increased sensitivity. [^{35}S]-dATP αS has been specifically formulated for use in 3'-end labelling of oligonucleotides for isotopic *in situ* hybridization.

■ Yeast genetics

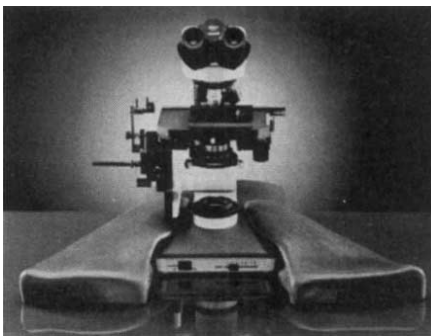
The Scienceware *Drosophila* sacrificing tool from Bel-Art Products is designed to provide an efficient, time-saving **system for cleaning large quantities of *Drosophila* bottles**, cutting cleaning times down to about an hour (*Reader Service No. 115*). This new tool is connected to the hot water supply.



Bel Art's *Drosophila* bottle cleaning tool.

The end of the stainless steel hot water tube dislodges the bottle cap and goes down to the bottom of the bottle where it breaks up the media pellet. Hot water sacrifices the flies. The handle is shaped so that it covers the mouth of the bottle allowing air to escape but not the *Drosophila*. The second tool has a double conical plastic scraper mounted on the tube and is also used with hot water. This tool helps to scrape the residual eggs and casings off the side of the bottle, as well as flushing out any residual pellets.

Micro Video Instruments announces a number of ergonomic improvements designed to enhance the utility of the Tetrad dissection microscope in yeast genetics (*Reader Service No. 116*). To reduce arm and wrist fatigue caused by long hours of tetrad dissection, MVI has added a ball bearing-style **micromanipulator** for precise, trouble-free operation and **moulded, wrap-around armrests** for added support



New levels of comfort for yeast geneticists.

and comfort. The armrests were specifically designed by Nikon for use with its Labophot-2 microscope and complement the ergonomic design of the Tetrad dissection microscope.

The new upgraded Tetrad II research **microscope for yeast genetics** from Carl Zeiss allows the researcher to use high magnification or fluorescence detection, as well as any low light-level contrasting technique with supporting photodocumentation (*Reader Service No. 117*). The microscope is a modified version of the Zeiss Axioskop FS (fixed-stage) microscope with advanced ICS (infinity colour-corrected system) optics. Special modifications in the Tetrad II version include a stage-mounted, vibration-free micromanipulator, with joystick control and adjustable movement ranges from 0.1 to 5 mm. The fixed stage has click stops at 5-mm intervals in both *x* and *y* directions, creating a coordinate system for reproducible spore transposition and documentation of spore location. An easy-to-read *x*-scale has also been added to the front face of the specially designed Petri dish holder. Removal of the micromanipulator turns the Tetrad II into the Axioskop FS full-fledged research microscope, which can be used in brightfield, phase-contrast, polarization, fluorescence, differential interference contrast, as well as video-enhanced contrast and video-intensified fluorescence imaging possibilities.

■ Hardware

Transporator Plus is the new **electroporation system** from BTX designed to produce the precise field strength and pulse length required for the transformation of bacteria and transfection of eukaryotic cells requiring a pulse length of five milliseconds (*Reader Service No. 118*). The system has its own power supply for convenience and is optimized for the electroporation of *Escherichia coli* and other bacteria. It can be used for construction of DNA libraries and to study gene expression for various proteins. Electroporation can also be used to insert metabolites and antimetabolites, such as ATP, into cells for studying cell activity. Protein introduction into cells for the study of the effects of restriction enzymes on cellular DNA and the introduction of antibodies to stain organelles used for studying cellular function are applications for which this instrument is particularly suited. The device is available with a repetitive pulse for flow-through or multiple pulsing applications; it can be used to electroporate a volume range from 40 μl to several litres.

Hoefler's compact PS3000 **power supply** has been designed to deliver the high voltage that is necessary for DNA sequencing and other applications such as isoelectric focusing (*Reader Service No. 119*). The power supply features three modes of operation: constant voltage, constant current or constant power. In each mode, Hoefler says

PRODUCT REVIEW

that regulation is good over the full range: 10 V to 3,000 V, 1 mA to 400 mA or 0 W to 200 W. The PS3000 has automatic mode switching, so if limits are reached in one mode, the power supply switches to a back-up limit mode to prevent overheating or damage to the instrument. Hoefer says that sensitive detection of ground leakage and open-circuit sensing increase operator safety. Two electrophoresis units can be run simultaneously from the parallel output jacks.



Hoefer's PS3000 power pack.

The new ISS UV **crosslinker** from Integrated Separation Systems uses ultraviolet energy to crosslink nucleic acids to membranes before hybridization (*Reader Service No. 120*). ISS says that the sensing circuitry



ISS's ultraviolet crosslinking device.

is based on 'true' UV monitoring, not combined sensing of white light and UV wavelengths as in some models; this prevents the photobleaching and photonicking that can occur with some 254 nm crosslinkers. ISS's model of crosslinker has 6 × 15-W bulbs and a 22 × 22 cm tray to hold the membrane.

To eliminate the problem of gel flattening associated with standard capillary transfers and to increase signal sensitivity of both Southern and northern blots, Schleicher & Schuell has developed the **turboblotter system** for doing downward alkaline and neutral transfers (*Reader Service No. 121*). The

device is designed to hold the appropriate gel blot papers and agarose gel in a format where the precut buffer wick can be placed on top of the gel, even with the surrounding buffer tray. S & S says that this arrangement allows wicking of buffer from two sides of the gel, resulting in an enhanced transfer efficiency. In addition to the reusable TurboBlotter device, blotter packs come with



S & S's turboblotter system.

7 × 8 cm to 11 × 14 cm precut 0.2 μm membranes and GB004 and GB002 wicking papers. The membranes are maximum strength Nytran for alkaline transfers and BA-S 83 supported NC for neutral transfers. Refill blotter packs are also available.

■ **Catalogues and custom services**
Oligonucleotides manufactured by Oligos Etc. are now offered in the United Kingdom through Semat (*Reader Service No. 122*). Oligonucleotides for use as inhibitors of specific gene expression, or oligonucleotides that bind to DNA in the nucleus blocking transcription of the target gene are available, together with a range of minor nucleotides, several deoxynucleoside analogues and a variety of modifiers. The triplex oligonucleotide range includes regular DNA linkages, as well as sulphur, Me-RNA groups, 5-methyl deoxy cytidine, 6-bromo deoxy uridine, biotin and psoralen.

Responding to customer demands, Lark Sequencing Technologies has introduced a **custom library screening service** (*Reader*

Service No. 123). Library screening can be a time-consuming procedure. Lark says that it has optimized existing protocols for restriction mapping and library screening and is in the process of developing more rapid and cost-effective protocols for these procedures. The choice of library, hybridization probe and screening strategy is determined after consultation with the customer; the company will either use a library supplied by the customer or purchase one from a commercial source. The customer can supply a cloned DNA fragment or synthetic oligonucleotide as a hybridization probe. If necessary, Lark says that it can design and synthesize an oligonucleotide probe based on existing sequence information. If the results of the primary screen are negative, Lark says that it will contact the customer to determine how best to proceed, perhaps by screening a different library. If potential positive clones are identified, the company will continue to isolate one or more clones and confirm their identity by Southern blot analysis or by primer extension sequencing directly from the isolated clone.

The 1993 **radiolabelled research compound catalogue** from Moravsek Biochemicals features 400 carbon-14 and tritium-labelled compounds for AIDS, cancer and other research fields (*Reader Service No. 124*). A complete structural formula index, storage recommendations, molecular weights and pricing are included. Custom tritium and carbon-14 labelling services are also described.

The new 1993-94 Fotodyne **molecular biology research products catalogue** features an array of new equipment and supplies (*Reader Service No. 125*). New items include the Visionary gel documentation system, GeneSprinter for rapid DNA sequencing, Economy transilluminators, Polaroid camera systems and film. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. For more information, fill in the reader service card bound inside the journal.

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